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THE PHYSICS OF SOUND WITH PARTICULAR RELATION TO EXAMINATION OF THE PATIENT

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INTRODUCTION

THE application of the principles of physics to the examination of the patient was introduced by Skoda and Hoppe[1] 100 years ago. Since that time their ideas have been amplified and experimentally verified.[2] We believe, however, this knowledge has been neglected in current teaching and for this reason we are presenting these ideas, not original, which are necessary for the student's appreciation of the fundamentals of physical diagnosis. Montgomery and Eckhardt's[3] admirable work clarified the physics of breath sounds, and herein we hope to accomplish the same for the phenomena of percussion and tactile fremitus. For clarity we shall present this material in three steps:

1. Sound waves—four qualities: intensity, pitch, duration, and quality (timbre).
2. Sound waves—four fates: diffusion, absorption, reflection, and resonance.
3. Application of the physical principles to tactile phenomena and percussion.

I

Sound is the result of rhythmic vibrations of the air striking upon the ear. The range of audible sound is from 16 to 36,000 vibrations per second. The range of musical tones is from 40 to 4,000. Sound waves can be represented diagrammatically to show the *four qualities* of sound: intensity, pitch, duration, and quality, as follows:

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1. *Intensity.*—In this we are concerned with the *amplitude* of the vibrations or the total excursion of the mass of air moved. In Fig. 1 wave length is the same in 1A and 1B, but amplitude differs. The tones shown are identical, but since 1A is greater in intensity than 1B it is a louder note.

2. *Pitch.*—In this we are concerned with *frequency* (vibrations per second) that differentiates one pitch from another. A deep note is one of low frequency, Fig. 1, 2A; a higher note is one of many vibrations per second, 2B.

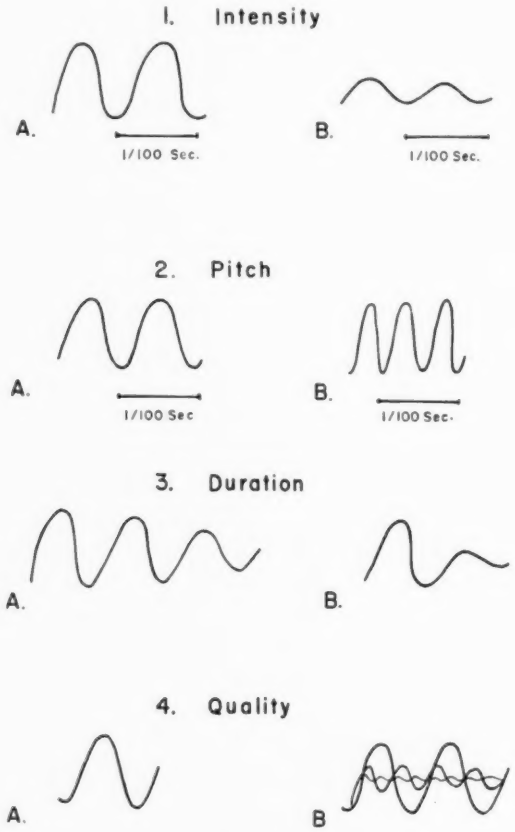

Fig. 1.

3. *Duration.*—All sounds diminish in intensity with time; they "die out." In Figs. 1 and 3, the tones are identical except that the duration of A is greater than that of B. Once the initial disturbance creating the sound no longer exists, the sound will persist for a shorter or longer time depending on a number of factors. The chief factor determining duration is the "sympathetic vibration" of a large mass of elastic material, usually air, which reinforces the original sound. For example, the plucking of a violin string alone produces a short sound, but with this string stretched over the sounding box, a large mass of air is set into vibration and a sustained tone results.

4. *Quality (timbre).*—The concept of quality is exemplified by the dissimilar tones of a violin, a voice, and a flute, all sounding the same note at the same intensity for the same length of time. Quality depends upon the presence or absence of overtones. When a violin string or a column of air vibrates at a certain frequency, the whole string or air column is in vibration as a unit.

The string or air column may vibrate in halves, giving a note of twice the frequency; or in thirds, fourths, and so on, and these various notes are heard superimposed upon the fundamental tone. What we usually call "richness" of tone is the presence of many overtones, all bearing a direct harmonic relationship to the fundamental, that is, all simple multiples of the fundamental. In Figs. 1 and 4, A is a pure tone, while B is rich in overtones.

II

Of what importance are these concepts when one is studying sound traveling through the body tissues? In tactile fremitus the vibrations originate within the body and travel outward to the palpating hands of the examiner; in percussion some of the sound waves travel from the percussing finger toward the interior of the body, where they are amplified or not, depending upon the properties of the substances underlying their point of entry; in auscultation the sounds arise within body tissues and travel toward the surface and the observer's ear.

Four fates lie ahead of such sound waves traveling through body tissues.

1. *Diffusion.*—The vibrations may set into motion tissues or air lateral to their direction of motion. Such lateral vibrations never reach the hand or ear of the examiner, since they gradually subside through diffusion in several directions.

2. *Absorption.*—This is the complete disappearance of sound vibrations, the energy of which is transformed into heat in the substance in which the sounds are damped out. Absorption must really be considered together with its opposite, transmission, since the properties of the medium determine whether sound will be transmitted or absorbed. A very elastic substance like air enclosed in a space with elastic walls (lung) transmits most of the sound waves passing through it. On the other hand very inelastic material (soft tissue, muscle) absorbes most of the vibrations.

3. *Reflections.*—The use of this term in connection with sound waves is similar to its use in optics. For reflection of sound waves to occur there must be two media meeting at an interface; reflection is favored by a large difference in density between the media. The best example of this is pleural fluid, which acts as a reflecting layer so that the vibrations of fremitus cannot reach the palpating hand nor can those of the percussion blow reach the underlying lung.

4. *Resonance.*—This term is used here to describe a physical principle, and not in its more specific meaning as one of the percussion sounds. The principle of resonance is that of tone reinforcement (sympathetic vibration) mentioned previously. If the physical properties of a large mass of tissue or enclosed air are such that it can be set easily into vibrations at a particular rate, then a sound of that frequency in the vicinity will cause the entire mass to vibrate. In this way the feeble sound of finger striking finger is amplified, as illustrated by resonance heard on percussion of the normal thorax or the tympanitic note heard over air-filled viscera.

III

We wish to stress the practical application of these fundamental concepts to the actual procedures by which we elicit and interpret the percussion note and tactile phenomena.

PERCUSSION

The purpose of percussion is to determine the physical properties of tissues by directing sound waves into them and observing the note which results. Since the object is to transmit the percussion blow to the underlying structures, *the pleximeter finger should be pressed very firmly* against the body wall. The plexor finger should strike a *sharp, staccato blow* and be immediately withdrawn to allow the fullest opportunity for vibrations to occur; a laggard blow may damp the vibrations. To offset diffusion (lateral radiation), however, *the percussion blow should be struck perpendicularly* to the surface, so that the resulting note will be affected only by the structures directly beneath. Skoda emphasized that percussion is chiefly comparative, and for comparing different areas the *intensity of the blow must be consistent.*

The question of the strength of the blow is somewhat dependent on the individual's reaction. For example, if percussion is performed too forcibly, organs cannot be well outlined because radiating sound waves impinge upon nearby organs or air spaces and confuse the resulting audible tone. This spreading occurs even under the best of conditions, as shown by the difficulty in outlining the right lower border of the heart. On the other hand, to outline masses lying at some depth, a rather heavy blow is required; for instance, the dulness over the left lower lung secondary to cardiac enlargement[4] is best appreciated with a forceful percussion.

It is evident that the percussion note will lie between two broad extremes; a large mass of air may be set into sympathetic vibration and so reinforce the percussion blow, or the sound may be reflected back at once by rigid, nonelastic structures, and only the dull thud of the percussion stroke will be heard. For convenience the sounds elicited are classified into four main gradations between these extremes. These will now be described in terms of their acoustic properties.

1. *Tympany* (Fig. 2, 1.).—Tympany is a definitely musical note, of pure pitch and with few overtones, produced when a mass of air vibrates in sympathy with the percussion note. It is best obtained normally over the region of the cecum or stomach. Whenever heard, it is an indication of the presence of air. Hence in pneumothorax, extreme emphysema, and over large cavities, it is a pathologic sign. Tympany can be approached experimentally by percussion over the normal thorax in full inspiration; such hyperresonance may also be simulated by percussion of a loaf of fresh bread or a feather pillow.

2. *Resonance* (Fig. 2, 2).—Resonance is a note of deeper pitch, richer in overtones and somewhat shorter in duration than tympany. It is the note heard over the normal lung. The elastic tissue of the alveolar walls tends to localize the vibrations and favors the setting up of these overtones by the separate vibration of many different air masses (alveoli).

3. *Dulness* (Figs. 2, 3).—Dulness is higher pitched and of shorter duration than resonance. A dense tissue underlies the pleximeter so that a comparatively small amount of air is set into vibration and the percussion note is only slightly reinforced. Dulness is the normal note over the heart, while over the lung it indicates consolidation, collapse, fluid, fibrosis, congestion, or a thickened pleura or chest wall. *Dulness in place of resonance is always a sign of disease.* "Ubi sonus est altior, ibi est morbus," as Auenbrugger said in 1755.[5]

4. *Flatness.*—Flatness is the epitome of dulness. The pitch is very high; the note is entirely nonmusical, rather a noise than a tone. The duration is minimal; the intensity is minimal; and the pleximeter finger even feels the sense of resistance. There is no reinforcement or sympathetic vibration whatever, and the note heard is simply that of the percussion blow itself. Flatness is the note heard normally over the thigh and pathologically over fluid and very thick tissue.

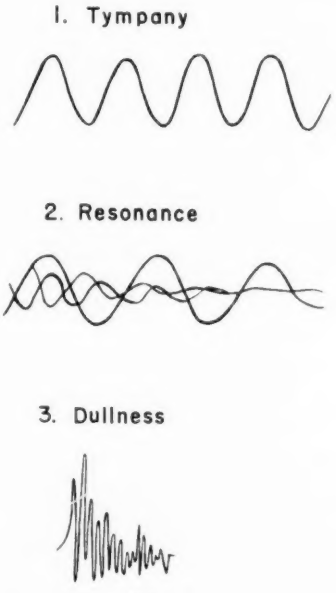

Fig. 2.

TACTILE PHENOMENA

To understand the principles underlying tactile fremitus, we drew the analogy of the human vocal cords, trachea, lungs, and thorax to a wind organ. The vocal cords correspond to the stops; the trachea to the pipe; the lungs to the bellows; the thorax to the resonator. The patient phonates in a full, deep voice, while the examiner's hands are placed on the thorax. Vibrations produced in the larynx pass down the air passages, are reinforced by sympathetically vibrating lungs, and are then transmitted through the pleura and chest wall to the examiner's hands. Fremitus therefore depends upon all the anatomic structures named, their condition, and particularly their physical properties with respect to the passage of sound waves (Fig. 3). Tactile phenomena arising from the movement of the heart, blood, pleural surface, or other viscera will follow the same principles in physics.

1. *Larynx.*—The vibrations of a bass voice correspond closely to the fundamental tone of the normal lung which they set into sympathetic vibration. The higher the pitch of the voice, the weaker are the vibrations one feels. This simple matter of voice quality must always be considered in evaluating fremitus. In weak, high-pitched voices one may feel little or no fremitus. In children, however, the higher-pitched voice corresponds to the fundamental tone of their lung and therefore fremitus is easily felt.

2. *Air Passages.*—The trachea and bronchi must be patent, or the vibrations will not pass freely and fremitus will be diminished or absent as in bronchial obstruction or in collapse of the lung with a closed bronchus. In atelectasis of the lung with an open bronchus the collapsed tissue may rest against the chest wall and so fremitus may be increased.

3. *Lungs.*—In consolidation fremitus is increased, because the solidified lung presents fewer air-tissue interfaces (alveolar septa) at which reflection can occur; and because solidified tissue vibrates more rapidly than does the normal elastic lung, thereby reinforcing the comparatively rapid vibrations of the spoken voice. In emphysema tactile fremitus is decreased because of larger air spaces and inelastic septa, which allow more absorption and diffusion of vibrations before they reach the palpating hand.

4 and 5. *Pleura and Pleural Space.*—These merely transmit the vibrations from lungs to chest wall. Any absorbing or reflecting layer will prevent such transmission; thus in thickened pleura, pleural effusion, and pneumothorax, fremitus is diminished or absent.

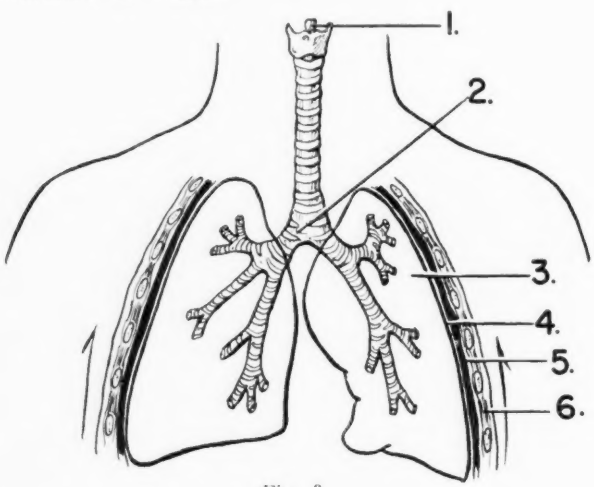

Fig. 3.

6. *Chest Wall.*—Fremitus is decreased over a fat or muscular chest wall because of absorption and diffusion; it is increased over a thin and bony one. One should also remember that for anatomical reasons fremitus is normally greater in certain locations than others. These locations are: (a) Posteriorly, over the right upper lobe, because the trachea lies in direct contact here with the lung apex, while on the left it is separated by the aorta, esophagus, and spongy connective tissue; (b) posteriorly low in the right interscapular area, because the right bronchus which is the direct continuation of the trachea points at this region; (c) in the interspaces rather than over the ribs. It is worth noting that the trachea does not run up and down, but is very oblique, running from the larynx posteriorly to the bifurcation and thence to each lung hilus, very near the posterior boundary of the mediastinum.

CONCLUSION

We offer this review as a guide to the student seeking to acquire skill in the physical examination of the patient. Such skill is not acquired by memo-

rizing lists of diseases and their physical signs, but rather by understanding the physics of sound in relation to normal and diseased tissues.

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EFFECT OF TUNGSTEN METAL DIETS IN THE RAT*

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FEW reports on the toxicity of tungsten metal are recorded in the literature, although the substance has been used as a substitute for bismuth salts in roentgenologic examination of the human gastrointestinal tract.

Bernstein-Kohan[1] killed and examined a rooster three days after he had fed the fowl 438.2 mg. tungsten daily for eight days. No trace of tungsten was found in the kidneys or the liver, but extravasations of blood were found in the mucous membrane of the duodenum, jejunum, and cecum.

Kruger[2] recommended powdered tungsten metal as a substitute for bismuth salts in roentgenologic examination of the gastrointestinal tract. He observed no ill effects in patients given orally 25 to 80 Gm. This followed preliminary work in which 3 rabbits were fed 3 to 5 Gm. of the powdered tungsten metal and 2 dogs were fed 10 to 20 Gm. In no case was there evidence of toxic action.

Waltner[3] incorporated 2 per cent tungsten metal in the McCollum stock diet and fed this to 1- to 2-month-old rats. He stated that growth was somewhat poor, but made no mention of control animals or of the duration of the experiment.

The present work was carried out in order to determine the effects upon the growth of the rat of extended feeding of various levels of powdered tungsten metal. These experiments should give evidence of any marked toxic action of the tungsten.

EXPERIMENTAL

Thirty-eight-day-old animals were chosen from the College colony of mixed Wistar albino and Minnesota piebald rats. Each group was made up of at least 5 females and at least 5 males with the sexes caged separately.

For the experimental diet, powdered tungsten metal† was mixed into the control diet of ground Purina dog chow. Containers of diet remained in the

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cages at all times, and a record was kept of the consumption. The rats were weighed at intervals of ten days for a period of seventy days.

At the end of seventy days, the animals were sacrificed and the gastro-intestinal tracts were examined grossly.

RESULTS

In Table I, averages for 5 males and for 5 females are listed separately for the control and the three experimental diets. The gain in weight, the diet consumption, and the tungsten (W) consumption, each expressed in grams, are listed for each successive 10-day period. The values for the entire 70-day experimental period are then summed.

TABLE I

DIET CONSUMPTION AND WEIGHT GAINED PER RAT
(AVERAGED FOR SUCCESSIVE TEN-DAY PERIODS*)

		CONTROLS		2% W DIET		5% W DIET		10% W DIET	
		MALE	FEMALE	MALE	FEMALE	MALE	FEMALE	MALE	FEMALE
10 days	Gain	38	26	45	41	46	35	47	29
	Diet	120	110	130	110	190	130	140	120
	Tungsten	0.0	0.0	2.6	2.2	9.5	6.5	14.0	12.0
20 days	Gain	36	27	28	22	45	20	37	20
	Diet	110	120	130	100	160	130	150	110
	Tungsten	0.0	0.0	2.6	2.0	8.0	6.5	15.0	11.0
30 days	Gain	40	21	37	15	39	22	41	17
	Diet	140	110	130	100	150	120	150	110
	Tungsten	0.0	0.0	2.6	2.0	7.5	6.0	15.0	11.0
40 days	Gain	24	13	20	12	23	10	27	9
	Diet	150	120	140	100	160	110	160	110
	Tungsten	0.0	0.0	2.8	2.0	8.0	5.5	16.0	11.0
50 days	Gain	22	14	21	8	27	10	24	11
	Diet	130	110	140	110	150	110	150	100
	Tungsten	0.0	0.0	2.8	2.2	7.5	5.5	15.0	10.0
60 days	Gain	14	3	9	11	17	7	11	4
	Diet	140	100	140	110	140	110	150	100
	Tungsten	0.0	0.0	2.8	2.2	7.0	5.5	15.0	10.0
70 days	Gain	9	6	12	5	10	3	11	3
	Diet	130	100	140	110	140	110	140	100
	Tungsten	0.0	0.0	2.8	2.2	7.0	5.5	14.0	10.0
Total	Gain	183	110	172	114	207	107	198	93
Total	Diet	920	770	1050	740	1090	820	1040	750
Total	Tungsten	0.0	0.0	21.0	14.8	54.5	41.0	104.0	75.0

*All values expressed in grams.

No extravasations of blood were evident in the mucous membrane of either the small or large intestine.

DISCUSSION

Over the seventy-day period, the three tungsten diets were without appreciable toxic effect on the males, if growth is used as the criterion. The males upon the 5 per cent and 10 per cent W diets actually showed greater total gains than did the control animals, while the males on the 2 per cent diet gained 6 per cent less than the controls. During the experimental period, each male on the 2 per cent diet consumed an average of 21.0 Gm. of tungsten; on the 5 per cent diet, 54.5 Gm. of W; and on the 10 per cent diet, 104.0 Gm. of W.

The female rats on the 2 per cent and 5 per cent diets showed normal gains despite consumption of 14.8 Gm. and 41.0 Gm. of W, respectively, over the

70-day feeding period. In the females on the 10 per cent diet each consumed a total of 75.0 Gm. of W and gained 15.4 per cent less than the controls. The 75.0 Gm. of tungsten could not be utilized for nutritional purposes, and since these rats consumed even less diet than did the controls, the reduced intake of nutritient material partially, at least, explains the smaller gain. It nevertheless is clear that the 2 per cent, 5 per cent, and 10 per cent W diets did not exhibit marked toxic action.

No extravasation of blood were evident in the mucous membrane of the small or large intestine. This differs from the findings of Bernstein-Kohan, but it is probable that the metal used in the present study was more finely and more uniformly powdered than that used by Bernstein-Kohan and thus mechanical injury was avoided.

It should be pointed out that in this study the growth of the rat is used as the main criterion of toxic action. It is not maintained that the growth study alone will reveal all possible toxic action.

SUMMARY

The feeding of 2 per cent, 5 per cent, and 10 per cent tungsten metal over a period of 70 days is without marked effect upon the growth of the rat, as measured in terms of gain in weight.

Each male of the 10 per cent diet consumed an average of 104.0 Gm. of W during 70 days and maintained normal growth. The females on this diet gained 15.4 per cent less than the controls and consumed 75.0 Gm. of W. This was the greatest variation from normal that was observed.

No extravasations of blood were observed in the gastrointestinal tract.

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SULFATHIAZOLE AND STAPHYLOCOCCUS ANTITOXIN IN EXPERIMENTAL STAPHYLOCOCCIC INFECTION*

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BEFORE the advent of chemotherapy with sulfonamide derivatives such as sulfathiazole and sulfadiazine, the literature on staphylococcic bacteremia had concerned itself mainly with forms of serotherapy and the use of bacteriophage. The recognition of exotoxins which were capable of producing many of the toxemic effects of staphylococcic suppuration prompted the use of sera containing adequate amounts of neutralizing substances against these toxins. These antitoxins have been found particularly useful when toxemia rather than bacteremia has been the important source of morbidity. As Spink, Hansen, and Paine[1] have pointed out, there are certain factors which vitiate the efficacy of such sera. These are in brief: (1) The organism may have invasive properties without much exotoxin production; (2) the exotoxin if produced may be neutralized by the serum without checking the inroads of the bacteria; (3) the usual serum though containing antihemolytic factors does not contain antileucocidin substances. We agree in the main with these cogent objections with the reservation that these methods served their purpose in the past. This was particularly so in suppurative processes with toxemia and only intermittent bacteremia. The effectiveness of serum was probably enhanced when other adjuncts were employed, such as bacteriophage, blood transfusion, surgical drainage, and general supportive measures. We have had no experience with Julianelle's rabbit serum, which contains specific carbohydrate precipitins.

The development of specific chemotherapy has changed the whole aspect of this problem, since it has provided the research worker and clinician with radicals capable of bacteriostatic effect even with the disadvantages inherent in all these compounds of lessened penetration into purulent foci. Spink et al.[1] report on the results of chemotherapy with sulfapyridine and sulfathiazole in staphylococcic bacteremia. Six of ten patients treated with sulfapyridine recovered; of nineteen patients treated with sulfathiazole, one died; one other patient died subsequently of myelogenous leucemia. They consider sulfathiazole superior to either sulfanilamide or sulfapyridine and emphasize the necessity of appropriate surgical drainage of abscesses. The use of combined methods of treatment is advocated by Skinner and Keefer,[2] based on a study of 122 cases of staphylococcic bacteremia. They recommend antitoxin, blood transfusion, surgical drainage of foci, and chemotherapy (sulfapyridine or sulfathiazole). They believe that recovery of the host depends on the presence of three conditions: (a) active and viable leucocytes and phagocytes; (b) antibacterial antibodies; and (c) antibodies to the toxins and leucocidin. Recently Kolmer and

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Brown[4] have reported on experimental chemotherapy and chemoserotherapy in staphylococcic infection. Mice were infected by intravenous injection of broth cultures; in addition, they also inoculated rabbits with broth cultures intravenously once a day for eight days in order to create a septicemic state analogous to clinical infection. Intradermal infection of rabbits was also produced to create spreading lesions due to toxin formation. They concluded that there was a slight but definite increment of therapeutic result with sulfathiazole and serum contrasted with sulfathiazole alone.

We have investigated the use of combined drug and serum therapy of experimental staphylococcic infection in a small but statistically significant group of mice. Sulfadiazine had not yet been described when the experiments were started, and we therefore confined our efforts to sulfathiazole alone and sulfathiazole combined with staphylococcus antitoxin.

TABLE I

CHEMOTHERAPY AND CHEMOSEROTHERAPY OF EXPERIMENTAL STAPHYLOCOCCUS INFECTION

TREATMENT	SINGLE DOSE (DRUG) GM./KG.	DAY OF DEATH													
None (control)	—	(2)	(2)	(4)	(4)	(4)	(5)	(5)	(7)	(8)	(8)	(13)	12		
Sulfathiazole	1.0	5	7	9	12	14	(15)	(18)	18	S	S	S	S		
Sulfathiazole and serum	1.0 0.5 c.c. of 1:2	4	4	(6)	10	(11)	13	17	18	S	S	S	S		
Serum	0.5 c.c. of 1:2	(3)	(3)	(5)	(6)	(7)	(7)	17							

S = survived.
Ringed numbers are animals showing renal abscesses.

METHODS

Male albino mice were injected intravenously by the dorsal vein of the tail with broth suspensions of a virulent strain of *Staphylococcus aureus*. Approximately 800 million organisms were given in volumes varying from 0.2 to 0.3 c.c. The strain was supplied to us through the kindness of Dr. G. W. Barlow of the Winthrop Chemical Co. and was of proved virulence to mice. We verified its pigment, hemolysin, and coagulase-producing properties. Sulfathiazole in a suspension of acacia was deposited into the stomach transorally with a blunt needle and syringe within 1½, 7, and 24 hours, then once daily thereafter. The dosage of the drug is given in Table I, and the usual bulk of material was 0.4 c.c. The absorption of the drug was proved by levels obtained on sacrificing a number of control mice and pooling the heart's blood. We were able to demonstrate a level of 15 mg. per 100 c.c. in 1½ hours, which, though not so high as figures obtained by van Dyke et al.,[5] were considered fairly adequate. Staphylococcus antitoxin was given in the rear gluteal muscles in doses of 0.5 c.c. of a 1:2 dilution at the same intervals as the drug. This amount corresponded to approximately 166 international units for a 20 Gm. mouse. The antitoxin was used without the drug as a control and also repeated in a small number of animals with the first dose given eighteen hours before infection. The reason for this will be given in the discussion of the results.

After death, animals were examined for abscesses in the kidneys particularly, as well as in other organs; cultures of the heart's blood were taken in those with indefinite or no abscesses and approximately one-half of the others. Survivors were observed for twenty-one days.

RESULTS

In all, 120 mice were used, of which 110 were selected in representative groups satisfactory for statistical purposes. Animals which had died accidentally due to trauma from the peroral injections were discarded.

In the entire group of animals studied, the mean survival time of controls was 6.7 days, whereas in the groups treated with sulfathiazole only and with sulfathiazole and serum, the mean survival times were 13.0 and 12.7 days, respectively. The survival time of animals which received serum only was 6.5 days; this is practically identical with that of the control animals. These figures were subjected to statistical analysis and are recorded in Table II. In a small representative series selected to indicate these factors more clearly, it is noted that one-third of the mice treated with drug only or with drug and serum survived (Table I). Life was definitely prolonged and abscesses noted in only two of those which succumbed. By contrast there were no survivors in the controls or in the serum-treated animals, and abscesses were almost invariably found.

TABLE II

CHEMOTHERAPY AND CHEMOSEROTHERAPY IN EXPERIMENTAL STAPHYLOCOCCUS AUREUS INFECTION: STATISTICAL ANALYSIS OF ENTIRE GROUP

TREATMENT	NO. OF MICE	MEAN SURVIVAL TIME (DAYS)	σ*	STANDARD ERROR OF MEAN
None (control)	30	6.7	2.24	.40
Sulfathiazole	30	13.0	6.7	1.2
Sulfathiazole and serum	30	12.7	6.7	1.2
Serum only	30	6.5	3.82	.84

*σ = Standard Deviation.

DISCUSSION

In common with other observers we found that sulfathiazole prolonged the life of mice infected with *Staphylococcus aureus* and prevented the occurrence of abscesses in the majority of such animals. The properties of this drug (as well as the more recently developed sulfadiazine) are all the more notable, since the massive experimental infection is in direct contrast not only to clinical infection but also to chemotherapeutic experiments upon pneumococci and streptococci. In the latter instance small numbers of virulent bacteria are used, so that quantitative mass relations between the chemical and the bacteria are in favor of the former with resultant sharp readings. The disappointing results with the combination of drug plus serum and with serum only require further elucidation. The potency of the commercial serum is assayed for antihemolytic action, and its dosage is standardized against the international antitoxin unit established by the League of Nations. It contains ample amounts of neutralizing bodies for alpha exotoxin, which is the important toxin in human infections; there is also a fair amount of beta-neutralizing antihemolysin. The latter hemolysin is the hot-cold hemolysin reported by most authors as

incapable of hemolyzing rabbit cells. Roy,[6] however, reports a hot-cold lysin which hemolyzes human as well as rabbit cells despite its resemblance to the beta toxin of Glenny and Stevens.[7] The serum also contains antibacterial bodies which should render it particularly useful in experimental animal infection. Under such conditions bacteremia is a more important issue than toxemia. The time of administration is a factor to be considered, since Farrell and Kitching[8] found that the highest level of absorption in mice did not occur until twelve hours after intraperitoneal injection and eighteen hours after subcutaneous injection. As indicated above, this interval was tested in addition to the method used in the main part of the experiment without materially influencing the results. In addition, such preliminary or prophylactic use of serum bears no resemblance to clinical conditions. We believe that such negative results do not necessarily invalidate the use of sera in clinical infection in which toxemia, longer time element, and reinforcement of natural immunity may tilt the scale in the direction of recovery. A suggestive factor in favor of the serum was noted, though it does not appear in the tables. Occasionally mice treated with drug and serum lived on for several days to a week after cessation of treatment, whereas those treated with drug only succumbed within a day or two after treatment was discontinued. One can speculate that the longer retention of serum created an increment of passive immunity in those mice which were not quickly overwhelmed by the infection.

SUMMARY

1. Sulfathiazole administered perorally prolonged the life of mice infected with *Staphylococcus aureus* and prevented the occurrence of abscesses in the majority of such animals.

2. The mean survival time of animals treated with sulfathiazole was 13.0 days. The mean survival time of those treated with sulfathiazole and staphylococcus antitoxin was 12.7 days. The mean survival time of those treated with serum only was 6.5 days which was practically the same as control animals which received no treatment. The results were further analyzed by statistical methods.

3. Animal experiments with staphylococcic infections do not entirely parallel clinical experience. In the latter certain factors which are discussed indicate that staphylococcic antitoxin may reinforce the immune processes of the host.

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CAROTID SINUS SYNDROME: A REPORT OF THREE ADDITIONAL CASES FROM THE CARDIOLOGICAL SERVICE, U. S. V. A. FACILITY, COLUMBIA, SOUTH CAROLINA

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HERING in 1923 conclusively demonstrated that reflex slowing of the heart might be brought about by carotid sinus pressure. Ample confirmation has followed. Particularly elucidative in this respect was the work of Heymans.[1] It was only in recent years, however, that these findings were made applicable to man. Weiss and Baker[2] in 1933 reported fifteen cases of an abnormally sensitive carotid sinus resulting in attacks of syncope and convulsions. Ferris, Capps, and Weiss[3] in 1935 reported an additional thirty-two cases. Since that time numerous similar cases have been reported and now the entity is universally recognized, but, unfortunately, the role it may play in the causation of syncope and unconsciousness is not always borne in mind. The purpose of this paper is to report three additional cases, which were not diagnosed until the patients were hospitalized.

GENERAL CONSIDERATIONS

It is generally recognized that the reflex arc lies within the autonomic nervous system. The afferent pathways lie chiefly in the intercarotid, the glossopharyngeal, and the hypoglossal nerves; in addition, the vagus and cervical sympathetic chains are also involved. Buey[4] has beautifully recorded the effects of intracranial section of the glossopharyngeal nerve on the blood pressure. In each instance of his four reported cases, a marked rise in blood pressure resulted from such section, and this rise persisted for several days.

The motor pathways are widespread and variable. This factor alone probably contributes largely to the varied clinical responses. Thus in man, the vagus nerve, the vasomotor depressor nerves, and the central motor pathways may be called into play, individually or collectively, and the resultant clinical entity will depend upon the predominance of any particular pathway. Thus, the three types of syndromes recognized have been a vagal type, characterized by cardiac slowing and asystole, followed by syncope and a convulsion; the depressor type, marked by a severe and often alarming fall in blood pressure; and the central type, in which no observable changes in blood pressure or heart rate take place to explain the syncope or convulsion. Commonly, the entity occurs in a mixed form and then it is most usual to classify it according to the most predominant finding. The pure depressor type is probably the least common, Weiss[6] having observed it in pure form in only two cases.

Signs and symptoms other than syncope are not uncommon. Abdominal manifestations are frequently seen, presumably due to a widespread autonomic discharge of the efferent limb of the reflex arc.[5] In order of frequency, fainting, dizziness and weakness, hyperpnea, pallor, bradycardia and asystole, numb-

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ness and tingling of the extremities, convulsions, drowsiness, epigastric distress, lacrimation, cough, amnesia, and palpitation are listed by Weiss.[6]

Local disease of the carotid sinus has been reported in the majority of cases, and as a group, the incidence of cardiovascular abnormalities was rather high in the reported cases. The average age lies in the fifth decade, but the youngest case reported by Weiss[6] was 13, and the oldest 74. The three reported cases here lie in the fifth decade.

It is a well-known fact that digitalis sensitizes the carotid sinus reflex, and it should be used with caution in elderly patients who complain of spells of weakness or dizziness. A practical point which might be remembered is the danger of its use in combination with anesthesia and manipulation of the neck in the operating room.

Stimulation of the carotid sinus is not wholly without danger. It is not wise to stimulate both at the same time. Marmor and Saperstein[7] reported a case of bilateral thrombosis of anterior cerebral artery following stimulation of a hyperactive carotid sinus, and Galdston[8] et al. reported two cases of thrombosis of the common, internal, and external carotid arteries, associated with hyperactive carotid sinus reflexes of the other side.

The treatment usually starts off as a medical problem, unless the attacks are so frequent and incapacitating that such treatment is not feasible. The general trend is an attempt at correcting all associated abnormalities wherever possible. Constricting collars should be avoided. Emotional upsets, worry, and fatigue should be minimized if at all possible. Reassurance of the patient with reference to epilepsy and organic heart disease is advisable. Specifically, the vagal type often responds to atropine sulfate, $\frac{1}{150}$ grain three to four times daily, orally; this will often prevent spontaneous attacks. Ephedrine, adrenalin, benzedrine, and paredrine may be used in conjunction, in an effort to irritate the ventricle, a state which is conducive to ventricular escape beats upon the occurrence of the usual sinoauricular block. The dose of any of the above drugs should be the minimum necessary to bring about the desired effect.

The depressor type may often be relieved with any of the sympathomimetic drugs, the longer acting ones being the more desirable. Ephedrine sulfate in doses of $\frac{3}{8}$ grain, three to four times daily, has been satisfactory. Benzedrine is probably longer acting and tolerated better by some. The dose ranges between 10 to 20 mg. three times daily. It is said that the side effects are less.

The cerebral type offers a more difficult problem. Here, prime attention must be paid to the associated pathologic condition. Any deficiencies must be treated along generally acceptable lines. Often surgical denervation offers the best hope in these cases. In any case, however, surgery may have to be resorted to if the medical therapy fails. The results have been good as a whole. Recurrences have been few, and an interesting point in this respect has been brought out by Weiss[6]; namely, that those patients in whom a severe, widespread neurosis, associated with a low basal metabolic rate is present, fare poorly, and such condition is considered by him a contraindication to surgical intervention.

CASE REPORTS

Case 1.—Mr. T. A. B., Reg. No. 28266, a 58-year-old white male, civil engineer by occupation, was admitted for the first time to the United States Veterans' Administration

Facility at Columbia, South Carolina, February 26, 1942, with an admitting diagnosis of "seizures, type undetermined." The family history was irrelevant. There was no history of epilepsy or other neuropsychiatric disorders in the family. Mother died at the age of 70 from "old age" and pneumonia. Father died at the age of 85. One brother died at the age of 35 of pneumonia.

The past history included the usual childhood diseases. He had typhoid fever at the age of 15, influenza in 1918, was hospitalized in 1932 for "arthritis." Venereal disease was denied.

He was seen by me February 27, 1942, after he had been referred for cardiac consultation by the neuropsychiatric service. The history obtained was as follows: There was no history suggestive of a diminished cardiac reserve, the patient denying exertional dyspnea, dependent edema, paroxysmal nocturnal dyspnea, and precordial or retrosternal pain related to exertion. He was not a known hypertensive. The first attack of syncope occurred in December, 1941, at which time he attempted to switch off the radio while in bed. This necessitated a movement of his head to the right, and it was followed immediately by an unconscious state, during which, his wife stated, both hands were drawn upward toward the shoulders. There was no convulsion, nor was there any incontinence, and he did not bite his tongue. The next attack occurred at 10:30 A.M., February 26, 1942, while attempting to put his right arm in the torn sleeve of his overcoat. That was all he remembered. He was observed to have fallen to the ground, injuring his face and left elbow. The right side of his tongue was cut.

Physical examination at the time of admission revealed a well-developed, well-nourished white male, weighing 158 pounds, 73 inches tall. There were bruises and abrasions of his face and elbow. The lungs were negative. Blood pressure was 128/80, the rhythm regular, and no cardiac enlargement was noted. There were no thrills or murmurs. Arcus senilis was present, and there was peripheral arteriosclerosis. Because of the suggestive history, a carotid sinus syndrome was suspected by the cardiologist, and, on February 27, 1942, a preliminary examination revealed the pulse to be 72, blood pressure 128/80, and the rhythm regular. While in the sitting position, stimulation of the right carotid sinus made the patient dizzy, and he keeled over. The blood pressure fell to 98/60. With this syndrome as the probable diagnosis, other possible etiological factors were investigated by the cardiological service.

The retinal vessels were within normal limits and there was no evidence of increased intracranial pressure. The discs were clear. The electrocardiogram revealed a regular sinus rhythm and a rate of 75. All QRS complexes were upright. The T waves were normal, and there was no significant ST deviation. The conduction was normal throughout. The interpretation was "within normal limits." X-ray of the skull revealed no abnormalities. X-ray of the chest revealed a thickening of the pleura and some coarse peribronchial thickening in the mid-portion and bases of both lung fields. The aortic, cardiac, and diaphragmatic shadows were normal. The red blood count was recorded as 3.69 million with 67.5 per cent hemoglobin on February 23, 1942, and 5.04 million with 73 per cent hemoglobin on March 3, 1942. The smear was not remarkable. The white blood count was reported within normal limits. The sedimentation index was 23 mm. per hour (Westergren). The urine revealed a specific gravity of 1.020 and was entirely normal. The Wassermann and Kahn tests were negative. Spinal tap was performed March 4, 1942. The fluid was clear, the pressure normal. The globulin test was negative; there were 2 cells per c.mm.; the dextrose was normal and the Wassermann reaction was negative. The Lange colloidal gold test was negative. Three basal metabolic rates were obtained, with readings of minus 13 per cent, minus 21 per cent, and minus 25 per cent reported.

On March 2, 1942, the syndrome was again tested. Pressure on the right carotid sinus resulted in syncope. The right side of the face was pulled upward and the patient lost all contact with his surroundings and fell over to the left. The blood pressure fell from 110/80 to zero?' The rhythm remained regular and did not slow.

On March 3, he was presented to the Clinical Pathological Conference, Dr. J. Heyward Gibbes of Columbia, presiding. Following stimulation of the right carotid sinus, the blood pressure fell from a level of 140/90 to 50/30, and the patient "passed out" completely. There was no change in pulse rate or rhythm. When stimulation was stopped, the

patient remained confused for a few moments and then returned to his normal state. Again the face was drawn upward on the right and he fell to the left. Pressure on the left carotid sinus failed to result in any abnormal reaction. He was placed on ephedrine sulfate, ¾ grain, twice a day, and given one grain of thyroid daily, receiving no other medication. He had no further spontaneous attacks while hospitalized. Subsequent attempts at causing syncope failed entirely. The patient gained nine pounds in weight and was discharged March 23, 1942, with instructions to continue the intake of ephedrine sulfate. The final diagnoses were:

1. Carotid Sinus Syndrome, Right, with Syncope, Depressor Type.
2. Arteriosclerosis, Radial and Brachial Vessels.

Follow-up: He again reported to me and stated that he suffered with a few more similar attacks, but admitted that he had not continued the ephedrine.

CASE 2.—Mr. W. L. S., Reg. No. 29612, a 46-year-old white male, was admitted for the first time to the United States Veterans' Administration at Columbia, S. C., on July 13th for the treatment of an upper respiratory infection. The family history was noncontributory, except that one sister had died at the age of 44 of Bright's Disease. He had the "usual" childhood diseases, and the remainder of the past history was irrelevant. Syphilis was denied. He used no drugs, nor did he use excessive amounts of alcohol.

The history revealed that the patient contracted an upper respiratory infection resulting in hoarseness and cough which began three weeks prior to admission. During these paroxysms of cough he became unconscious, having had nine such attacks in the preceding three weeks. Two such attacks occurred during the first two weeks of admission.

Routine admission examination revealed a blood pressure of 146/76 and some arteriosclerosis of the radial vessels. A special eye, ear, nose, and throat examination by the consultant revealed an acute catarrhal pharyngitis and laryngitis. He was seen by the neuropsychiatrist, who found nothing of relevance.

Blood and urinary work-up were not remarkable. The Wassermann and Kahn tests were negative. Spinal fluid was normal and the pressure was not increased. X-ray of the skull revealed three unexplained areas of rarefaction in the frontal region. X-ray of the chest was negative. Electrocardiogram revealed left axis deviation, some slurring of the QRS complexes and low T waves in all leads. No conduction defect was noted. The rhythm was normal sinus.

He was seen in consultation by the Cardiological Service, at which time the following was noted: He had enjoyed good health and had no diseases of relevance until eight weeks prior to admission, at which time he "caught a cold" and suffered severe paroxysms of coughing. A tentative diagnosis of whooping cough was made by his local physician. He was not a known hypertensive, was never a congestive failure, and there was no history of coronary insufficiency. The first attack of syncope followed a coughing paroxysm and occurred while the patient was in a sitting position. He lost consciousness (he was told this) for a few minutes. There was no generalized convulsion, no loss of sphincteric control, and he did not bite his tongue. He aroused in a few moments. Eight or nine similar attacks followed, and each was brought on by a short coughing paroxysm. No voluntary movement of the head or neck could precipitate an attack.

The relevant findings in the cardiovascular examination revealed a normal-sized heart with a soft systolic blow over the aortic area. A-2 and P-2 were approximately equal. The pulse was 72 and the blood pressure 110/70 in the sitting position. The radial and brachial vessels were soft and compressible. Visualization of the fundi revealed mild tortuosity of the vessels. There was no evidence of failure.

On August 11, 1942, stimulation of the right carotid sinus caused a typical syncopal attack. There was a complete asystole; no heart sounds at all were heard, and the blood pressure fell to zero. With this definite clue, the entire procedure was repeated and a simultaneous first-lead electrocardiogram was obtained. With the patient in a sitting position, stimulation of the left carotid sinus for about thirty seconds caused the patient to lose consciousness. The eyeballs rolled upward and he fell over to the left side. Recovery was rapid following cessation of stimulation. Complete asystole followed a slowing of the heart rate (Fig. 1).

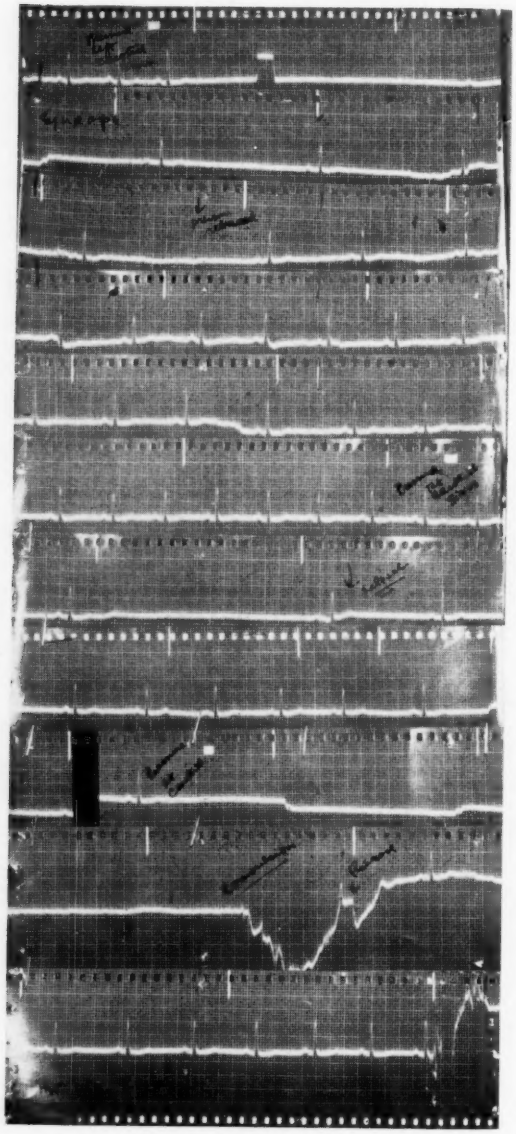

Fig. 1.—Patient W. S. Continuous tracing, Lead I, showing the effect of carotid sinus pressure. At the top of this tracing three complexes are noted, and at the indicated point, pressure was applied on the left carotid sinus. Patient fainted, and the pressure was not applied steadily and constantly. Note that following the asystole there are four contractions recorded, originating in the S-A node. At the arrow the pressure was released and cardiac activity was resumed. At first it was slow and later at a more rapid rate. Pressure on the right carotid sinus was applied, as indicated, and again there is a long pause, followed by an occasional contraction. Pressure was released at the point indicated and then reapplied on the right carotid sinus. This was followed by a complete asystole and syncope and finally a convulsion, at which point the pressure was again released. It is noted that there are no nodal or ventricular escapes.

A short while later the right carotid sinus was stimulated, and pressure for about thirty to forty seconds resulted in loss of consciousness and a short clonic convulsion, which lasted as long as stimulation was continued and ceased soon after the cessation of stimulation of the right carotid sinus. The blood pressure fell, secondarily to the asystole.

The diagnosis was: carotid sinus syndrome, bilateral, with syncope, of the vagal type.

The patient was placed on atropine sulfate, $\frac{1}{150}$ grain three times a day, and was discharged soon thereafter, having been advised to continue this medication. He has not been heard of since his discharge on September 19, 1942. Whether or not the upper respiratory infection was the sensitizing factor and the cough the trigger mechanism is unknown. Certainly it appears likely that such was the case.

CASE 3.—Mr. L. S., Reg. No. 29796, a 52-year-old white male, was admitted for the first time to the United States Veterans' Hospital, Columbia, S. C., July 29, 1942, complaining of fainting spells of three years' duration. The family history was noncontributory. He had the "usual childhood diseases," typhoid fever in 1912, and denied any venereal infection.

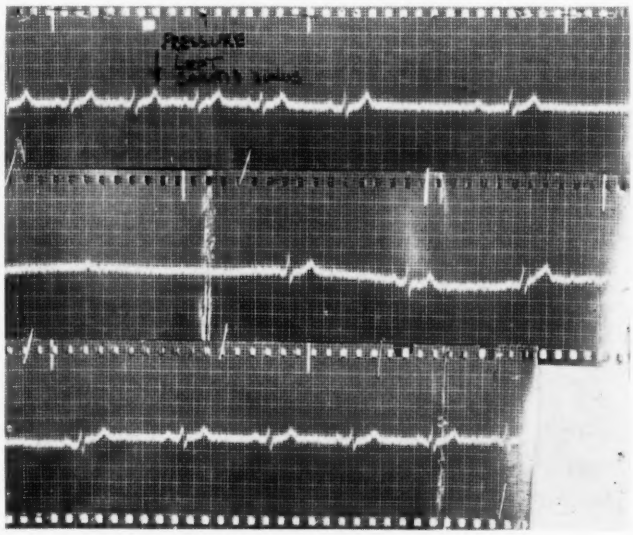

Fig. 2.—Patient L. S. Pressure on left carotid sinus, at the indicated point, resulted in, first, a slowing of the normal sinus rhythm, and then a complete asystole, followed by syncope. The point of release of pressure is not indicated on this tracing, but it is noted that upon cessation of asystole the normal sinus rhythm reappeared, at first slow, and then at a more rapid rate.

The history given to the admitting office revealed that he had "spells," consisting of "shaking and swishing noises in his head," complete blindness, and falling to the ground, for three years prior to admission. He stated that exertion brought on these attacks. Consciousness was regained soon after falling to the ground. He also complained of weakness, stating that he lost 30 pounds during the two months prior to admission. He stated that once the shaking had started, he could prevent the rest of the spell by holding his head still between both hands. The remaining history was irrelevant.

Examination at the time of admission revealed a blood pressure of 120/80. The heart tones were recorded as faint, but there was no enlargement, no murmurs, nor arrhythmia. The remainder of the examination was negative, including a neurological examination.

The blood, urinary work-up, including modified Mosenthal, phenolsulfonphthalein output, nonprotein nitrogen determination, and microscopic urine examinations, were within normal limits. The Wassermann and Kahn tests were negative. Spinal fluid was crystal clear, under normal pressure, and negative in all other respects. The colloidal gold test was negative. X-ray of the skull, lumbosacral spine, and hips was normal. X-ray of the chest was negative. Four-lead electrocardiogram was within normal limits.

He was seen in consultation by the Cardiological Service on August 18, 1942, at which time further history revealed that these attacks could be brought on by unusual movements of the head. The frequency varied, sometimes occurring as often as two or three times per week, but usually not so often. There was no history of bladder or rectal incontinence; he never bit his tongue; and he was never told that he had a generalized convulsion during any of these attacks. At times he said he felt as though a fly were in his left ear. No history was obtained of coronary insufficiency nor of pre-existing hypertension.

Cardiac examination revealed no increase in heart size, regular sinus rhythm, and no thrills or murmurs. The first apical sound was recorded as "poor." A-2 and P-2 were equal. There was no peripheral sclerosis and no evidence of congestive failure. The retinal vessels were normal.

Pressure on the right carotid sinus did not slow the heart rate nor reduce the blood pressure, and resulted in no clinical manifestation of syncope. Very little pressure on the left carotid sinus, of short duration, resulted in complete asystole, followed by syncope. (See Fig. 2.)

A clinical diagnosis of carotid sinus syndrome, left, of the vagal type, with syncope, was made. He was placed on atropine sulfate, by mouth, in doses of $\frac{1}{150}$ grain, for a period of three weeks, had no recurrence, and was discharged on September 2, 1942. He has not been heard from since.

SUMMARY

1. Three cases of carotid sinus syndrome, two of the vagal type and one of the depressor type, are reported. In all, medical treatment afforded some relief. One of the patients with the depressor type had an associated low basal metabolic rate. His response to medication was not reliable, possibly in view of his failure to diligently take the ephedrine. The results with the vagal type cases have been more encouraging.

2. In all three of these reported cases the presence of the syndrome was easily established. In two the history revealed the precipitating factor, to be an abnormal or sudden movement of the head and neck. In the third, the patient learned that he could avoid such an attack by holding his head still between his hands.

3. The syndrome should be borne in mind in all cases of unexplained syncope, especially if associated with abnormal movements of the head and neck.

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ADDENDUM

Since the above paper was submitted for publication, three more cases of carotid sinus syndrome of the vagal type were observed.

CASE 1.—The first was noted in a 60-year-old Negro male, a railroad worker by occupation, who had an attack of unconsciousness of unknown cause about six years ago, followed by numerous attacks of giddiness and "swimming sensation in his head." No further attacks of outright syncope occurred and on the outside he was diagnosed as having coronary disease. When seen, he presented marked hypersensitivity of the right carotid sinus, resulting in asystole and syncope. He was a known hypertensive, and electrocardiogram revealed evidences of myocardial disease. Because of the fact he was not admitted for treatment, further observation was impossible.

CASE 2.—The second case noted was in a 53-year-old white male, a textile worker by occupation, who was admitted because of bilateral deafness of the perception type, with degeneration of the right auditory nerve. He had no spontaneous attacks of syncope, and, when routinely examined on the cardiac service, he presented a very definite bilateral carotid sinus syndrome of the vagal type, with asystole and syncope. At present, he is being treated with atropine sulfate, grain $1/150$, orally, three times daily. He has not been observed sufficiently to determine whether or not this will be effective. Aside from some arteriosclerosis of the peripheral and retinal vessels, cardiological work-up was essentially normal. He returned subsequently, having had three spontaneous attacks of syncope. His response to atropine was poor, and he will in all probability come to surgery.

CASE 3.—A 72-year-old white male, a known hypertensive and coronary arteriosclerotic cardiac patient, entered with a history revealing three recent, spontaneous syncopal attacks of unknown etiology. The case proved to be one of extremely sensitive left carotid sinus syndrome of the vagal type with asystole and syncope. Atropine and ephedrine, in large doses, have prevented further spontaneous attacks.

THE INFLUENCE OF MUSCULAR WORK AND FATIGUE ON THE STATE OF THE CENTRAL NERVOUS SYSTEM*

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INTRODUCTION

THE reciprocal relationship between muscular fatigue and fatigue of the central nervous system has theoretical interest and practical application. Most industrial occupations involve muscular work as well as functions of the central nervous system. Hence, the development of general fatigue in industry depends on the influence of both systems on one another. It would seem that knowledge of this relationship could be useful in military affairs, since there are many occupations and situations in which fatigue of the central nervous system contributes to general fatigue arising out of muscular strain or effort. Concerning this question, no experimental material is available, so that Ivy[1] in a recent review states only, "As is well known, heavy muscular exercise influences mental performance because of the tendency to fall asleep." It would be practical to know what amount of muscular work is necessary to produce a depressing effect on the central nervous system, how long the effect lasts, and whether the type of work is an influencing factor. From a theore-

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tical point of view it would be interesting to investigate the mechanism of the depressing effect. While the depression of the central nervous system by muscular fatigue has been generally accepted as a fact on the basis of subjective experience, little is known of the effects of short, moderate muscular work on the state of the central nervous system; i.e., whether short, moderate muscular work exerts a stimulating effect. The fusion frequency of flicker seems to be a method suitable for the study of the influence of muscular work on the state of the central nervous system. The fusion frequency of flicker is that rate of successive light stimuli necessary to produce an effect equal to continuous illumination. It depends on chronaxie, latent period, and refractory period of the visual system (retina, visual pathways, visual centers); therefore, it indicates the excitability of the visual system, and perhaps, to a certain degree, the excitability of the whole central nervous system. It has been repeatedly demonstrated that general fatigue develops first in the sensory centers.[2] The fusion frequency of flicker is depressed in fatigue of the central nervous system,[3, 4] in pathologic fatigue of patients with hypothyroidism,[5] or circulatory insufficiency;[6] on the other hand, the fusion frequency of flicker increased parallel to the relief of fatigue in patients with hypogonadism after treatment with testosterone,[7] and in normal subjects after benzedrine[8] and pervitin.[9] The fusion frequency of flicker can be measured with great accuracy, and cannot be influenced by effort or by training. The effect of muscular work on the central nervous system probably depends on the physical condition and sensitivity or resistance of the central nervous system of the individual subject. Therefore, equal effects of a given standard type of work cannot be expected to result in different people. Short, moderate exercise might produce a depression of the excitability of the central nervous system in a weak subject, while a rather strenuous muscular effort might fail to do so in a stronger subject. Hence, it would seem to be more proper to express the results of this study in terms of distribution of deviations in a group of 54 subjects. Each type of work might increase the excitability of the central nervous system in some subjects and depress it in others. But a shift from prevailing stimulating effects in the majority of subjects after short, moderate exercise to prevailing depressing effects after more strenuous exercise may be expected. Four different types of work were investigated:

1. Static work, where fatigue is rather localized.
2. Short, moderate exercise (30 genuflexions), without fatigue.
3. Pulley, for three minutes or until fatigue.
4. Running until fatigue.

Although the performance of these different types of work cannot be compared on a quantitative basis, it is clear that the effort increases from Type 1 to Type 4.

METHOD

The fusion frequency of flicker was measured under standard conditions of surrounding illumination, illumination of the test patch, visual angle, light-dark ratio, and exposure. Direct current was used for the light source. Details of method and procedure are described in an earlier publication.[3] The

fusion frequency of flicker was measured before the exercise and ½, 1, 2, 3, etc., minutes after the exercise. The fusion frequency was also measured during the static exercise, where the subject remained in the same sitting position as used for the determination of the fusion frequency. In the other types of exercise, it was not possible to do so.

Types of work.—(a) Static exercise: The subject had to hold a load of 8 pounds with the right arm straight in horizontal position until completely fatigued.

(b) 30 genuflexions were performed within one minute according to the rhythm of a metronome. This exercise did not produce fatigue, so that the subjects would have been able to continue.

(c) Horizontal pull with both arms of a pulley weight of 26 pounds at a rate of 20 per minute for women and 30 per minute for men. The stop signal was given after three minutes, but 16 subjects ceased work earlier because of fatigue.

(d) Running: The subjects were asked to run through a corridor of 33.5 meters' length back and forth until fatigued. Although they were urged to run fast, there were great individual variations of speed (between 1.43 and 4.19 meters per second), total distance (from 201 and 737 meters), and time (from 70 to 300 seconds). There was no inverse ratio between speed and duration, so that the individual differences must be explained by different physical and training condition, and perhaps by different willingness to exhaust themselves. If the subject did not break off the exercise earlier, the stop signal was given after 670 meters (Nos. 6, 17, 19, 20, 29, 33, 39, and 44), in one subject (No. 8) after 737 meters, in order to avoid too great differences in the amount of work performed. One subject (No. 16) broke off the performance after 80 seconds because of side pains, which occurred also in a second investigation after several weeks' interval. Although the running exercise cannot be regarded as standardized, it was for each subject by far the hardest exercise test, compared to the other tests, "a", "b", and "c". Only one exercise test was investigated at a time, so that there was an interval of several weeks between the different exercise tests for each subject. The investigations were performed at the same time (1½ hours after lunch). All results are given as deviation from the initial value before the exercise.

SUBJECTS

The 54 normal subjects (25 men, 29 women) ranged from 18 to 38 years. The average age of the male subjects was 33.3 years, that of the female subjects was 27.4 years. All passed through a medical examination, and none showed any abnormal findings. The height of the group of male subjects varied from 5 feet, 1⅞ inches to 6 feet, 1¾ inches, the weight from 130 to 207½ pounds. The correspondent values for the group of women are: Height 5 feet, 2 inches to 5 feet 9½ inches, and weight from 111 to 141½ pounds. They were employed in sedentary occupations. Only a few (4 men, 1 woman) were in a training condition which could be considered as above the average, i.e., they were participating rather regularly in athletic games or exercises; the others did so only occasionally, at greater intervals, or not at all. In this respect the

TABLE I

EFFECT OF FOUR TYPES OF MUSCULAR WORK ON THE FUSION FREQUENCY OF FLICKER, EXPRESSED IN NUMBER AND PERCENTAGE OF DEVIATIONS RELATED TO THE TOTAL NUMBER OF CASES

1 TYPE OF WORK	2 TOTAL NO. OF CASES	3 ONLY INCREASE	4 INCREASE AND DECREASE	5 ONLY DECREASE	6 ALL INCREASES	7 ALL DECREASES	8 WITHOUT CHANGE
(a) Static Exercise	53	42 = 79.2%	7 = 13.2%	3 = 5.6%	49 = 92.5%	10 = 18.8%	1 = 1.9%
(b) Genuflexions I	54	34 = 63.0%	12 = 22.2%	6 = 11.1%	46 = 85.2%	18 = 33.3%	2 = 3.7%
Genuflexions II	47	31 = 66.0%	10 = 21.3%	5 = 10.6%	41 = 87.3%	15 = 31.9%	1 = 2.1%
(c) Pulley	49	20 = 40.8%	18 = 36.7%	10 = 20.4%	38 = 77.5%	28 = 57.2%	1 = 2.0%
(d) Running	52	2 = 3.8%	11 = 21.2%	39 = 75.0%	13 = 25.0%	50 = 96.2%	

experimental group may be regarded as a fairly good average of normal population.

During the period of investigations several subjects dropped out, so that the total number of subjects was 53 for the static exercise, 52 for the running exercise, and 49 for the pulley exercise. At the end, test "b" (genuflexions) was repeated on 47 subjects. Thus, the experimental material covers about 2,500 determinations of the fusion frequency.

RESULTS

Table I shows the effect of the four types of muscular work on the fusion frequency of flicker, expressed as frequency of positive (stimulation) and negative (depression) deviations, absolute as well as in percentage of the total number of cases (column 2). Column 3 shows the number of cases who responded to exercise with only an increase, column 5 those who responded only with decrease, while column 4 shows the cases where increase as well as decrease were observed during the recovery period. Column 6 shows all cases with increase, i.e., the sum of columns 3 and 4, while column 7 shows all cases with depression, i.e., the sum of columns 4 and 5. Only in five experiments, muscular exercise (genuflexions, pulley exercise) failed to produce a definite response; in all other experiments the deviations exceeded the experimental error. Table I shows that any type of exercise may produce an increase of the fusion frequency of flicker in some subjects and a depression in others, but there was a pronounced shift of their distribution. The number of cases who responded only with increase (column 3) decreased from 79.2 per cent in static exercise to 63.0 and 66.0 per cent in genuflexions, to 40.8 per cent in the pulley exercise, and to 3.8 per cent in the hardest type of exercise (running). Accordingly, the number of cases who responded only with depression (column 5) was lowest in the static exercise (5.6 per cent) and highest after running (75 per cent). The same inverse relationship can also be seen in columns 6 and 7. While increase after static exercise and genuflexions occurs in 85 to 90 per cent of all cases, a depression is observed in 96.0 per cent after running. These results show that muscular exercise may increase as well as depress the excitability of the central nervous system; the factors responsible for the stimulating effect prevail in short, moderate exercise, while the depressing factors prevail in hard exercise. In intermediate types of exercise, such as the pulley exercise, 36.7 per cent show increase as well as decrease. The error of the percentage of increases (column 6) and decreases (column 7) was calculated according to the formula $\pm \sqrt{\frac{P_1\% \cdot P_2\%}{n}}$ where P_2 per cent $= 100 - P_1$ per cent (Poll[11]).

For the static exercise, the error of the percentage of all increases was ±3.618; that of the percentage of all decreases was ±5.367. The corresponding values for Genuflexions I were ±4.832 and ±6.404, respectively; for Genuflexions II, ±4.873 and ±6.783, respectively; for the pulley exercise, ±5.965 and ±7.069, respectively; for the running exercise, ±6.005 and ±2.699, respectively. The limit of statistical reliability is regarded to be three times these values. This means that the prevailing increase of the fusion frequency after static exercise

TABLE II

LIMITS, AVERAGE, AND DISTRIBUTION OF MAXIMUM DEVIATIONS OF THE FUSION FREQUENCY OF FLICKER, IN PERCENTAGE OF THE TOTAL NUMBER OF OBSERVATIONS, AFTER FOUR TYPES OF MUSCULAR PERFORMANCE

TYPE OF WORK	1	2	3	DISTRIBUTION 4											
	MAX. DECR.	MAX. INCR.	AVER.	−10 TO −8.1	−8 TO −6.1	−6 TO −4.1	−4 TO −2.1	−2 TO −0.1	+2 TO +0.1	+4 TO +2.1	+6 TO +4.1	+8 TO +6.1	+10 TO +8.1	+12 TO +10.1	OVER +12.1
(a) Static Exercise	−4.4	+13.2	+3.84			3.4	6.8	6.8	13.5	28.9	25.4	8.5		5.1	1.7
(b) Genuflexions I	−5.6	+14.0	+2.46			3.1	14.1	10.9	12.5	28.1	18.8	9.4	1.6		1.6
Genuflexions II	−5.8	+13.2	+2.97			3.6	10.9	12.7	14.5	36.4	7.3	5.5	5.5		3.6
(c) Pulley	−8.8	+20.8	+0.97	1.5	1.5	7.5	19.4	13.4	17.9	23.9	5.9	5.9	1.5		1.5
(d) Running	−9.6	+ 8.0	−3.77	3.2	14.5	30.6	24.6	6.4	6.4	6.4	6.4	1.6			

and genuflexions and the prevailing decrease after running are statistically significant. On the other hand, the prevailing of the percentage of increases after the pulley exercise is only a tendency without statistical significance. Significant are also the high percentage of increases in types "a" and "b," compared to the low percentage in type "d"; and the high percentage of decreases in type "d," compared to the lower percentage in types "a" and "b." It is interesting that the amount of work producing a depression of the fusion frequency is rather small; in three subjects the speed of running was slow (subjects 1, 12, and 49), or the distance was small (subjects 3, 12, 21, 22, and 49); nevertheless, a distinct depression was observed. Only a rather short, moderate type of work produces increase of fusion frequency in the majority of normal subjects. There was no direct relationship between the frequency or the magnitude of the increase in types "a" and "b" and the training condition. For instance, genuflexions failed to produce any distinct deviation in one of the best trained subjects (No. 8). It is possible that the "moderate" type of exercise was too slight for this subject to provoke any response of the central nervous system.

Table II shows the magnitude of the deviation of fusion frequency after muscular work. Only the maximum deviation is considered. Column 1 shows the maximum depression of the whole group; column 2 shows the maximum increase. The lowest depression is –4.4 after static exercise and increases to –9.6 after running. Occasionally also, moderate types of work may produce marked depression. The upper limit of increase (column 2) is smallest after running (+8.0). The distribution (4) is given in intervals of deviation from –10 to –8.1, from –8.0 to –6.1, etc., as percentage of the total number of observations. The distribution of the values in intervals of distinct, but not extreme, responses, such as –4 and –6, and +4, reflect the tendency much better than the stray values of extreme limits (columns 1 and 2). The percentage of observed depressions in the intervals –4 and –6 increases from type "a" to type "d," so that for type "d" depressions ranging between –2.1 and –4.0 are about four times as frequent as for type "a," and this is even more manifest for the interval between –4.1 to –6.0 deviation. Type "b" (genuflexions) was repeated after an interval of three months. For the second investigation only 47 subjects were available. The limits, averages, and distribution of increase and depression are similar in the first (Genuflexions I) and second (Genuflexions II) investigation. This shows that the distribution of values is rather constant and characterstic for the given type of exercise.

Table III shows the duration of deviations of the fusion frequency, subdivided into two groups with durations of less or more than eight minutes. The time of eight minutes is arbitrary. The duration of the increase exceeds eight minutes in ten to fourteen cases in types "b" and "c," while five cases after static exercise and only one case after running show a duration of increase exceeding eight minutes. The lower incidence of longer lasting increases after static exercise may be explained by the comparatively small amount of work performed in this type of exercise. Also, the duration of decrease is less than eight minutes in all cases after static exercise, and in 28 of 31 observations after Genuflexions I and II. There are almost as many cases with depressions ex-

ceeding eight minutes as those less than eight minutes after pulley exercise, while the depression lasts longer than eight minutes in the majority of cases after running. In several cases, the values were still distinctly depressed sixteen and nineteen minutes after the exercise. A short, hard exercise may depress the excitability of the central nervous system for a rather prolonged time.

No relationship was found between speed, time, and distance in running and the magnitude of deviations. The deviations apparently depend at least as much on individual constitutional factors as on the amount of work performed.

TABLE III

DURATION OF DEVIATIONS OF FUSION FREQUENCY OF FLICKER AFTER MUSCULAR WORK

TYPE OF WORK	DURATION OF INCREASE		DURATION OF DECREASE	
	LESS THAN EIGHT MINUTES	MORE	LESS THAN EIGHT MINUTES	MORE
(a) Static Exercise	43	5	9	–
(b) Genuflexions I	35	11	16	2
Genuflexions II	27	14	12	1
(c) Pulley	28	10	15	13
(d) Running	11	1	15	35

Fig. 1 shows two typical responses for each type of exercise: The short, but pronounced, increase during and after static exercise, the prolonged depression after running, and the wavelike reaction after pulley exercise. The response after running is also wavelike; however, with the level below the resting value, while the waves oscillate around the resting value after pulley exercise. Wavelike reactions also were observed on an elevated level after genuflexions, although less frequent.* It appears that stimulating as well as depressing factors are present during the whole period of recovery after exercise, reaching their maximum at different times.

Our material (25 men, 29 women) allows the comparison of performance as well as of response of the fusion frequency between men and women. The comparison of performance between men and women has some interest because the material on this question is scanty. A difference of the average between the group of men and the group of women was regarded to be significant when exceeding the value of $2\sqrt{SE_1^2 + SE_2^2}$. The average duration of static performance in 25 men was 53 seconds, that of 28 women only 27 seconds. The difference (26 seconds) was highly significant, the value of the expression being only 6.672.

The pulley exercise was stopped after three minutes, so that no statistical comparison of endurance is possible. The male subjects worked at a rate of 30 per minute, the women at the rate of 20 per minute, so that the performance of women was only $\frac{2}{3}$ that of men. There is some evidence, however, that the endurance of women was inferior also in this type of work. Out of 25 men, only three stopped working before three minutes because of fatigue, while 13 out of 24 women did so, although they worked at a slower rate.

In regard to running exercise, the performance of women was inferior in all respects (distance, speed, duration). The subjects were instructed to run

*Allen and Schwartz[10] observed wavelike responses of the fusion frequency of flicker after stimulation of the sense of taste, smell, and after acoustical stimulation.

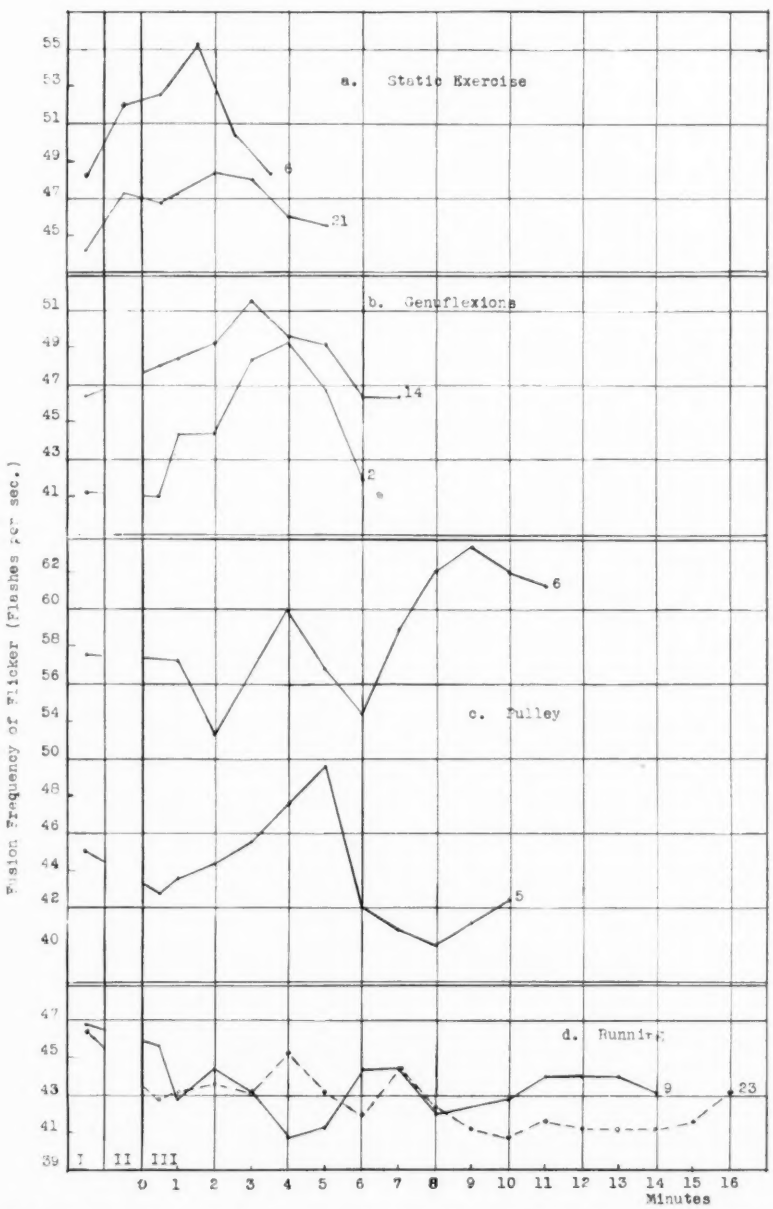

Fig. 1.—Typical effect of four types of muscular work with increasing effort (*a*, static exercise; *b*, genuflexions; *c*, pulley exercise; *d*, running) on the fusion frequency of flicker (flashes per second). "I" means the initial fusion frequency before the exercise; "II" means the period of exercise; during this period the fusion frequency was measured only in type a; "III" means the recovery period after the exercise. The subjects, from whom the curves are taken, are indicated by their numbers. The typical response after short, moderate exercise (types a and b) is an increase of the fusion frequency, while hard exercise (type d) produces prolonged depression. The fusion frequency is still below the initial level, fourteen and sixteen minutes after the exercise. Moderately heavy work (type c) produces increase as well as drop of the fusion frequency.

TABLE IV

INFLUENCE OF SEX ON THE MAXIMUM DEVIATION OF THE FUSION FREQUENCY OF FLICKER AFTER 30 GENUFLEXIONS

1 Exercise	SEX	2 NO.	3 ONLY INCREASE	4 INCREASE AND DECREASE	5 ONLY DECREASE	6 ALL INCREASES	7 ALL DECREASES	8 WITHOUT CHANGE	9 AVERAGE	10 DIFF.	$2\sqrt{SE_m^2 + SE_f^2}$
Genuflexions I	Men	25	19 = 76.0%	3 = 12.0%	2 = 8.0 %	22 = 88.0%	5 = 20.0%	1 = 4.0 %	+3.14		
	Women	29	15 = 51.7%	9 = 31.0%	4 = 13.8 %	24 = 82.7%	13 = 44.8%	1 = 3.4 %	+1.87	1.27	
Genuflexions II	Men	24	19 = 79.1%	3 = 12.5%	2 = 8.35%	22 = 91.6%	5 = 20.8%	0	+3.58		
	Women	23	12 = 52.3%	7 = 30.4%	3 = 13.1 %	19 = 82.7%	10 = 43.5%	1 = 4.35%	+2.33	1.25	
Total	Men	49							3.36		
	Women	52							2.08	1.28	1.399

fast, but the speed was not regulated. The average distance men ran until fatigue was 598 meters; that of women 331 meters. The difference (267 meters) was significant ($2\sqrt{SE_1^2 + SE_2^2} = 66.224$). The average speed of men was 282 cm. per second; that of women, 230 cm.; the difference (52 cm. per second) was significant (value of the expression: 20.72). Also the duration was significantly lower in women. This corroborates results of Metheny et al.,[12] who found the average endurance in running at constant speed on a treadmill half as much in a group of 17 women as in a group of 30 men.

In all types of exercise, "a," "c," and "d," the performance of women was smaller than that of men. No significant difference of the response of the fusion frequency of flicker could be seen in these types. It must be assumed, therefore, that the smaller amount of exercise elicited the same response in women as the greater amount of exercise did in men. In type "b" (genuflexions) the amount of work was the same for men and women. The average deviation of the fusion frequency of flicker after the exercise is +3.36 in men and +2.08 in women (total of all experiments, Table IV). The difference (+1.28) is somewhat smaller than the value of the expression (1.399), but both values are almost equal. Although the difference is not quite significant, there is an outspoken tendency to a higher increase of the fusion frequency in men than in women. The same tendency is reflected in the distribution of increase and decrease; 76 and 79 per cent of men (Series I and II, respectively) showed only increase (column 3) after the exercise, while only 52 per cent of the women reacted in the same way. Correspondingly, 20 per cent of men and 44 per cent of women showed a decrease of the fusion frequency (column 7).

DISCUSSION

There are four possible factors responsible for the deviation of the fusion frequency of flicker after muscular exercise.

1. *Circulation.*—The minute volume of blood flow increases during exercise, including the blood flow to the brain. In moderate exercise the oxygen demand of the brain is probably only slightly increased, so that the oxygen supply to the brain exceeds the oxygen demand. This improvement of oxygen supply is likely to increase the fusion frequency of flicker.

2. *Oxygen Debt.*—The huge oxygen debt after hard exercise expresses a general oxygen want including the brain tissue, i.e., the general oxygen demand exceeds the oxygen supply. This deterioration of the oxygen supply is apt to decrease the fusion frequency of flicker.

It is clear that factor "1" will prevail after moderate exercise, factor "2" after hard exercise such as running. This would explain the prevailing increase of fusion frequency after moderate work and the prevailing decrease after hard work.

3. *Radiation of Stimulation.*—A radiation of voluntary impulses from the motor centers to other parts of the central nervous system is generally assumed. This radiation, for instance, is believed to be responsible for the early stimulation of the respiratory center in muscular exercise. The existence of impulse radiation can be shown by the early increase of fusion freuqency during static exercise (Fig. 1). The fusion frequency increased twenty seconds after the

onset of static exercise in 83 per cent of all observations. This time is too short to account for any circulatory factor. Radiation of stimulation should be assumed also in the other types of exercise, although it cannot be as readily demonstrated because of the longer duration of exercise and the later time of measurement of the fusion frequency. Although radiation of impulses can be demonstrated only in the early phase of exercise, before the possible interference of circulatory factors, there is no reason to believe that their existence is restricted only to that period. According to Allen and Schwartz's results, reflex stimulation or inhibition of the fusion frequency of flicker might be observed for several minutes after acoustical stimulation.

4. *Radiation of inhibition.*—It seems logical to assume a radiation of inhibition as well as radiation of stimulation from the motor centers. According to Wedenski[13] and Ukhtomski,[14] strong stimuli are likely to produce and spread inhibition to other parts of the nervous system, while moderate stimuli are likely to radiate stimulation. This, too, would explain the distribution of prevailing stimulation after short, moderate exercise and of prevailing depression after running.

The coexistence of nervous and circulatory factors would account for the complicated form of reaction (Fig. 1) after muscular exercise.

In earlier communications we have shown that subjective fatigue in types of work without muscular effort parallels rather closely the depression of fusion frequency of flicker. There is no reason to expect such close relationship between subjective fatigue and drop of fusion frequency after muscular work, because of the interference of muscular fatigue. Static exercise "a" was continued to subjective fatigue; nevertheless, it produced an increase of the fusion frequency of flicker, probably because the fatigue is localized in a rather small part of the central nervous system and because the increase of the oxygen demand is very slight. Certainly, the response of the fusion frequency of flicker does not depend only on the type and amount of exercise, but also on the state and condition of the central nervous system. In other words, the reaction to the same type of work will vary in the same subject when the condition of the central nervous system is altered. We have a chance observation to support this view. In a male subject of 37 years the pulley exercise was investigated two days after he had lost his father. He was in a state of depression and fatigue (due to the loss of sleep during several preceding nights). The exercise depressed the fusion frequency by −5.4 flashes. The test was repeated after two months, when the subject had overcome his depression and was in normal condition. At this time, he responded to the exercise with an increase of +5.2 flashes, which lasted for more than eight minutes.

From a practical point of view, these results indicate that a hard exercise of rather short duration may depress the state of the central nervous system for a comparatively long period of time. This explains the depressed capacity for mental work or other occupations involving nervous functions on the background of physical fatigue. Perhaps, this also accounts for the increased accident rate in fatigue, because of the probable deterioration of alertness. It can be concluded that in occupational work involving both muscular and nervous functions diminution of muscular fatigue will probably diminish

fatigue of the central nervous system. Short, moderate exercise may improve the state of the central nervous system. This would explain the favorable effect of gymnastics during rest pauses observed in several industrial plants. About 10 per cent of normal men and about 20 per cent of women, however, would probably react with depression even after short, moderate exercise. Therefore, a selection for this procedure is advisable. The selection could be made by using the fusion frequency of flicker.

SUMMARY

The reactions of the fusion frequency of flicker of 54 normal subjects after four different types of exercise of increasing severity ("a," static exercise; "b," 30 genuflexions; "c," pulley exercise; "d," running) were studied. Each exercise produced definite deviations of the fusion frequency of flicker. The majority of normal people (about 80 to 90 per cent) respond with an increase of the fusion frequency after static exercise and 30 genuflexions, and with a decrease of the fusion frequency after running. After pulley exercise, about 40 per cent of the subjects respond with both increase and decrease. The magnitude and the duration of the depression correspond to the severity of exercise. These results may explain the depressing effect of physical fatigue after severe muscular exercise, and the stimulating effect of short, moderate exercise on the state of the central nervous system. The endurance of women in static exercise, pulley exercise, and running is significantly lower than that of men, while there is no significant difference of the reaction of the fusion frequency of flicker. The response of the fusion frequency of women after an equal amount of work (30 genuflexions) appears to be inferior to that of men.

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THE SIGNIFICANCE OF ERYTHROCYTIC PSEUDOAGGLUTINATION*

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PSEUDOAGGLUTINATION is a term used by Wiener[1] to designate all types of erythrocyte aggregation likely to cause confusion in the clinical determination of blood grouping. Included under this term, he lists rouleaux formation and the aggregation of erythrocytes which may be found in aged whole fluid blood ("autoagglutination").

The same term, pseudoagglutination, was used by Barnard[2] in connection with a certain type of aggregation observed when erythrocytes are acted upon by weak alkali. This type of aggregation appears to be due to a saponification of the cholesterol-lecithitides of the stroma causing the cells to stick together. It appears probable now that the "autoagglutination" of Wiener and the aggregation of rH-positive erythrocytes by the agency of anti-rH factor is of a similar nature, that the phenomenon may be produced by a variety of agents, and that pseudoagglutination, as latterly described, is a precursor to lipolytic hemolysis. For these reasons, it is suggested that the term, pseudoagglutination, be reserved for the specific prehemolytic process to be described, and for the purposes of distinction and that of classification pseudoagglutination may be compared with the other, better-known forms of erythrocytic aggregation; i.e., (1) rouleaux and (2) specific hemagglutination.

1. *Rouleaux.*—This is a physiologic process, having a definite teleologic role in the maintenance of capillary circulation. The mechanism underlying rouleaux is a physical one, as admirably demonstrated by the experiments of Kegerreis.[3] Rouleaux masses are unidimensional, the cell aggregates forming only by apposition of their biconcave surfaces, and the phenomenon, as would be expected from its physiologic nature, is entirely reversible. While this form of aggregation may be seen macroscopically, its designation, as rouleaux, is a microscopic procedure.

2. *Specific Hemagglutination.*—The mechanism of hemagglutination is immunologic. The aggregates are tridimensional, the clumps forming by indiscriminate apposition of groups of cells. Thus in an agglutinated mass, a single cell may be in contact simultaneously with many others, while in rouleaux, no erythrocyte may be in aggregated contact with more than two others. Hemagglutination is a completely irreversible process, and the agglutinated cells are morphologically and structurally altered as evidenced by their susceptibility to hemolytic agents. The designation of an aggregation phenomenon as a true agglutination may be made microscopically, but it is even more characteristic, when complete, on macroscopic examination.

3. *Pseudoagglutination.*—The mechanism appears to be chemical and to consist of a saponification of the erythrocyte envelope. Thus, it can be pro-

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duced by dilute alkalis or, more rapidly, by toluene. The role of the latter, as a hemolytic agent, is by reaction with the cholesterol-lecithitides of the stroma. (Svedberg and Nichols,[4] 1927; Barnard,[5] 1940.) The cell membrane becomes sticky, and where erythrocytes come into juxtaposition, they tend to adhere. Since the greater proportion of contacts will be at the circumferential surface, pseudoagglutinative aggregates tend to be bidimensional. This is seen best in microscopic preparations with alkali as previously described,[2] and particularly if there are some cotton fibres in the field, the tendency of the cells to adhere to such fibres by their edges is clearly shown. At the same time, changes in the cell morphology under the influence of the hemolytic agent is evident; the cells swell, lose their biconcave shape, and gradually disappear along with liberation of the contained hemoglobin.

TABLE I

	DIFFERENTIATION OF ERYTHROCYTIC AGGREGATION		
	ROULEAUX	PSEUDOAGGLUTINATION	HEMAGGLUTINATION
Mechanism	Physical	Chemical	Immunologic
Aggregates	Unidimensional	Bidimensional	Tridimensional
Reaction	Reversible	Reversible or Irreversible	Irreversible
Change in Cell Morphology	None	Plasmolysis	Crenation
End Result	None	Hemolysis	Hemolysis (in vivo)
Best Determined	Microscopically	Microscopically	Macroscopically

By adjusting the concentration of alkali in which the erythrocytes are immersed, it is possible to demonstrate varying degrees of reversibility for this process. Extremely dilute alkali (1 to 3000 NaCN) will induce a type of aggregation remarkably similar in both microscopic and macroscopic aspects to that produced in rH-positive erythrocyte suspensions by anti-rH sera. Such aggregates may be broken up, after their formation, by vigorous agitation. Again, if such a suspension is allowed to settle in a test tube at room temperature, or if it is centrifuged, all cells are aggregated into a solid mass, but if the treatment be not too prolonged, the cell masses may be broken up by agitation. Since this sequence may be duplicated frequently in reaction mixtures of rH-positive cells and anti-rH sera, the concordance in their behavior between two supposedly diverse agents leads to the belief that, if pseudoagglutination is a precursor to hemolysis, the anti-rH factor is a hemolysin rather a true agglutinin. It remains to prove the major premise; the pseudoagglutination is indeed such a precursor, and to this end we have studied the pseudoagglutination produced by dextrose and that appearing spontaneously in aged, stored blood.

THE EFFECT OF DEXTROSE ON ERYTHROCYTIC AGGREGATION

Pendleton[6] reported an agglutinative action of concentrated dextrose or sucrose solutions on human erythrocytes. Such an action, he thought, might explain certain reactions occurring during the intravenous infusion of dextrose solutions. At the Cook County Hospital, we have routinely employed dextrose as a storage medium for blood for transfusion and had definitely demonstrated

that concentrations of dextrose up to 5 per cent (higher than could ever be sustained in vivo) were without any obvious effect on stored blood or its erythrocytes.* We had in fact demonstrated that somewhat higher concentrations of dextrose (12 to 20 per cent) completely inhibited the agglutination of erythrocytes by their own type-specific serum and that this inhibition was reversible in that the cells could be agglutinated when the concentration of dextrose was reduced. The inhibition of the interaction between specific hemagglutinin and hemagglutininogen (for this is what the action of dextrose proved to be) was masked in dextrose concentrations above 20 per cent by the phenomenon that Pendleton described, a phenomenon which has all the characteristics of pseudoagglutination and is invariably followed by hemolysis.

PSEUDOAGGLUTINATION OF ERYTHROCYTES IN AGED, STORED BLOOD

The blood bank has afforded the opportunity for observations on a large number of samples of stored, citrated dextrose-whole blood with regard to their tendency toward pseudoagglutination and hemolysis. While there is a considerable difference between individual samples in the rate at which either of these phenomena make their appearance, there is a distinct correlation between the two in any single specimen. After from one to twenty days of refrigeration, without agitation, the corpuscular layer will be a dark blue-violet color. While the supernatant plasma may show no evidence of hemolysis, it may have been proceeding for some time in the corpuscular layer, and the evidence thereof may have been confined to the corpuscular layer because of the low rate of diffusion of hemoglobin. If the cells in such a sample be suspended by gentle agitation, on their resettlement, free hemoglobin will be evident in the plasma. Such samples show many pseudoagglutinative aggregates.

Since hemolysis is present in these samples and may escape detection, except by allowing an inspection of the plasma after resuspension of the corpuscles, the need for inspection, in this manner, of stored whole bloods, before they are used for transfusion, is obvious.

In the main, samples of blood which showed early hemolysis would likewise show early pseudoagglutination, and this parallelism is carried over to bloods which reacted positively to the serologic tests that are supposed to be diagnostic of syphilis. Naturally these samples were never used for transfusion, even for a known syphilitic prospective recipient (on the theory that although it was not possible to give an individual more syphilis than he already has, he might be given a different kind), and consequently were subject to observation over a long period of time. Curiously enough, in serologically positive blood samples, both pseudoagglutination and hemolysis appeared to be markedly delayed.

It is true that many bloods exhibited gross hemolysis which seems to have occurred spontaneously in the stored samples without showing any pseudoagglutination, and the converse is also true: that samples exhibiting marked pseudoagglutination might show no greater tendency to hemolyze in the stored condition than unaltered bloods. In certain samples, the corpuscles will not mix diffusely with the plasma on agitation, but will swirl around in large tailed,

*It has been noticed, however that even 5% dextrose accelerates or facilitates the appearance of hemolysis in pooled, incompatible bloods.

discoid masses, the bidimensional nature of which is apparent macroscopically. These masses we formerly referred to as "clots" until they were found to be devoid of fibrin. The fibrinogen was still present in undiminished amount in the plasma, and these aggregations showed, microscopically, all the characteristics of pseudoagglutination. Before the nature of this aggregation was appreciated; because the erythrocytes could be separated by continued agitation and because the samples showed little or no hemolysis at this stage, some of these bloods were used for transfusion. The practice was discontinued because of the high incidence of severe hemolytic reactions.

From a practical standpoint, therefore, the presence of pseudoagglutination in a blood sample precludes the use of that sample for transfusion even though hemolysis may be absent. The pseudoagglutinated erythrocyte must be regarded as a potentially hemolyzed one, and the clinical significance of pseudoagglutination is identical to that of specific hemagglutination in incompatable blood.

SUMMARY

The term pseudoagglutination is restricted to a phenomenon observed in erythrocytes which have been acted upon by lipolytic hemolysins. Criteria are set up for its distinction from rouleaux and from specific hemagglutination.

Toluene, high concentrations of dextrose, and anti-rH factor action produce pseudoagglutination, which likewise appears spontaneously in stored whole blood.

Stored blood samples which show positive serologic tests for syphilis appear to be more resistant to spontaneous pseudoagglutination and hemolysis than do serologically negative bloods.

Pseudoagglutination is a precursor to, or a manifestation of, the initial stages of hemolysis by lipolytic agents.

Blood samples which show pseudoagglutination, even without manifest hemolysis, are unsuitable for transfusion.

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THE OCCURRENCE OF A DOUBLE ZONE PHENOMENON IN ANTIHUMAN TISSUE SERUM*

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INTRODUCTION

DURING a group of experiments in which unperfused normal human kidney was injected into ducks, the duck serum was found to contain antibodies against human blood serum. This was not an unexpected result since the original material (antigen) contained some whole serum. Preliminary precipitin tests, however, showed such a well-defined double zone it was considered important to clarify its occurrence and if possible relate it to the proteins known to exist in human serum.

MATERIALS AND EXPERIMENTS

Normal human kidneys from acutely fatal trauma cases, in persons under the age of 40, showing no gross or microscopic changes beyond a mild degree of cloudy swelling, were removed within four hours of death; capsules stripped, and the kidney cut in long thin sections and frozen for twenty-four hours. After the pelvic fat was removed and the kidney ground fine, the pulp was suspended in minimal normal saline. Following an eight- to twelve-hour fat extraction (ether) in the cold, the water solution was precipitated with an excess of 80 per cent cold acetone. This precipitate, when resuspended in normal saline, proved fairly soluble. This precipitate was used to inject intravenously (3 c.c. to 4 injections) and intramuscularly (4 c.c. increasing to 10 c.c., 5 injections) in continuity every third day into adult Canton ducks. The fowls were bled on the tenth day following the last injection; the sera from 4 ducks were pooled and titrated against the following: (1) whole human blood precipitate (prepared similarly to the kidney precipitate except it had no fat extraction), (2) normal human sera, (3) whole *serum* precipitate (acetone precipitate in saline suspension), (4) erythrocyte cell stroma material, (5) hemoglobin, (6) globulin-free (Na_2SO_4 precipitated) serum. This wide variety of blood constituents was utilized in hopes of finding out what blood constituent was brought down in each precipitin zone. Results are given in Tables I, II, and III. Explanation of tables:

The antigen dilutions were made by mixing the antigen with normal saline, and diluting. The tests were run in 3 mm. diameter tubes, first putting the duck serum in and then the antigen with a capillary pipette. The tubes were water bathed four hours at 37.5° Centigrade and iceboxed over night. Readings were made in a reflected light against a concave mirror. In spite of attempting to layer the solutions, they did mix to some extent. Results are read from 0 to ++++ and where there was a ring it is shown thus ++°.

*Read by title, before the American Association of Pathologists and Bacteriologists, 42nd Annual Meeting, St. Louis, Missouri, April, 2, 1942.

This work was done while the author was Director of the Montgomery County Laboratories. At present, Passed Assistant Surgeon (R), U.S.P.H.S., Bethesda, Md.

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TABLE I

WHOLE HUMAN BLOOD PRECIPITATE (ANTIGEN) TITRATED AGAINST ANTIHUMAN DUCK SERUM

DIL. OF ANTIGEN	1.	2.	3.	4.	5.	6.
.030	0					
.021	0					
.020	0	+++		+	0	+
.019	0					
.018	0	+++		+	0	++
.016	0					
.015	0	++++	++++	++	++	+++
.014	0					
.013	0	++++	++++	+++	+++	+++
.011	0					
.010	+	+	+++	++	+	+++
.009	+++	+++	++++	++	++++	+++
.007	+	++	++++			+
.005	0	++				0
.003	0	+		+	+++	0
.002	0					0
.0019	0	0				++°
.0017	+					++
.0015	+					+++
.0013	++++					+++
.0011	++++		++++			+°
.0009	+++	+	++++	+	+++	+°
.0007	++	0		+	++	0
.0005	0			0		0
.0003	0			0		
.0001	0					

TABLE II

NORMAL HUMAN SERUM AND SERUM PRECIPITATE SUSPENSION TITRATED AGAINST ANTIHUMAN DUCK SERUM

DIL. OF ANTIGEN*	1.	2.	3.	DIL. OF ANTIGEN†	1.	2.	3.	4.
.030								
.021								
.020	0	++	+		0	0	0	+++
.019								
.018	0	++	++		0	0	0	+++
.016								
.015	0	+++	++°		++	0	0	+++
.014								
.013	0	++	++°		++++	++++	++++	+++
.011								
.010	+	0	++°		0	++++	++++	++++
.009	++	0	+++°		0	++++	++++	++++
.007	++	++	0		0	+++	+++	+++
.005	++	0	0		++++	+++	+++	++
.003	++	0	0		++++	++	++	++
.002	0	0	0		+++	+	+	0
.0019	0	+°	0		++	0	0	0
.0017	0	+°	+++		++	0	0	0
.0015	++	+°	++°		++	0	0	+
.0013	++	+°	++°		++	+++	+++	+
.0011	0	+°	++°		0	+++	+++	0
.0009	0	+°	+°		0	0	0	0
.0007	0	+°	+°		0	0	0	0
.0005	0	+°	+°		0	0	0	0
.0003								
.0001								

*Normal human serum.
†Whole serum precipitate.

TABLE III

ERYTHROCYTE STROMA, HEMOGLOBIN, AND GLOBULIN-FREE SERUM TITRATED AGAINST ANTIHUMAN DUCK SERUM

DIL. OF ANTIGEN*	1.	2.	3.	DIL. OF ANTIGEN†	1.	2.	3.	4.	DIL. OF ANTIGEN‡	1.	2.	3.	4.
.030	0												
.021													
.020	0	+++	0		+	++°	0	+		+°	0	0	0
.019	0												
.018	0	+++	+		+	++°	0	++		+°	0	0	0
.016	0		+										
.015	0	+++	+		+	++°	+°	+°		0	0	0	0
.014	+		+										
.013	+	+++	+		+	+°	+°	+°		0	0	+°	0
.011	+												
.010	0	+++	+		++	+°	+	++		+°	0	+°	0
.009	0	+++	+		+++	+°	+	+		+°	0	+°	0
.007	0	++	+		++	0	+°	+		+°	0	0	+°
.005	0	0	0		0	0	+°	+		+°	0	+°	+°
.003	0	0	0		0	0	+	0		+°	+°	+°	0
.002	0	0	0		0	0	0	+		0	0	+°	0
.0019	0	0	0		0	0	0	0		0	+°	+°	0
.0017	0	0	0		0	0	0	0		+°	0	0	+°
.0015	0	0	0		0	+	0	0		+°	+°	0	++°
.0013	0	0	0		0	0	0	0		+°	+	0	+++°
.0011	0	0	+		0	0	0	+		+°	+	+°	++°
.0009	0	0	0		+	0	0°	0		+°	+°	0	0
.0007	±	0	0		±	0	0°	0		0	+	0	+
.0005	0	0	0		0	0	0	0		0	0	0	+
.0003	0		0										
.0001													

*Erythrocyte stroma.
†Hemoglobin.
‡Serum albumin.

DISCUSSION

The duck serum tested contained a group of major antibodies against kidney substance, which are not discussed here, and minor antibodies. These minor antibodies, in varying dilutions, seemed to contain two entities, each with its distinctive optimal zone of precipitation. When the duck antihuman serum was matched against whole blood precipitate, serum precipitate, or normal serum, the double zone made its appearance. Red cell stromas (possibly globulins) gave a single precipitin zone as did hemoglobin. Their precipitins occurred in the zone of lower dilutions. Contrariwise, a globulin-free serum gave positive precipitins in higher dilutions. The precipitin reactions as seen from the tables were not always too clear cut, indicating that the precipitin method is more sensitive than the physical and chemical methods used to divide the compounds. As nearly as can be ascertained from published chemical data, the serum globulins are the larger, heavier molecular weight compounds, whereas, serum albumins are the smaller weight molecules.* These data, when fitted into the present precipitin system, show the larger molecular compounds combining in the lower dilutions, the smaller molecular weight compounds combining in the higher dilutions. Even though the antibodies are in the same solution, optimal zones of precipitation make their separation possible. The method may be useful in separating out unwanted antibodies where specific tissue-antibodies are to be studied.

*Svedberg, T., and Eriksson-Quensel, I. B.: Tabulae Biological Periodicae, V (4), 352 (1935-36). Quoted from Schmidt, C. L. A., The Chemistry of the Amino Acids and Proteins, Springfield, Ill., 1938, Charles C Thomas.

SUMMARY

Because of optimal precipitin conditions, it was possible to discover, in the minor antibody group of duck antihuman kidney serum, a double zone phenomenon. This phenomenon appears to rest upon the differential chemical structures involved. The higher molecular weight substances, the globulins, combine in the zone of lower dilutions. The smaller molecular weight albumins combine in the higher dilutions.

HEMATOLOGY OF THE PERIPHERAL BLOOD AND BONE MARROW OF THE DOG*

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WITH THE TECHNICAL ASSISTANCE OF DOROTHY BLAIR

NORMAL values for peripheral blood of dogs have been reported by a number of investigators[1-13] (and others); however, the available data is still comparatively small. Only three reports of differential bone marrow counts were found.[14-16] In the course of several investigations considerable data have been obtained on normal values for peripheral blood of the dog together with some observations on bone marrow, and we are presenting a summary of this data.

METHODS

Adult mongrel dogs of both sexes weighing 8 to 25 kg. were used and all received our standard kennel ration. The animals were trained to be tied down and were allowed to remain quiet for some time before withdrawing the blood samples, which were obtained from the femoral artery at approximately the same hour of the morning. A mixture of dried ammonium and potassium oxalate was used as the anticoagulant. The blood was drawn into Thoma certified pipettes and diluted with Hayem's solution for the erythrocyte counts, and 1 per cent acetic acid solution tinted with gentian violet for the leucocyte counts. After hand shaking the pipettes for at least three minutes, a few drops were discarded, the hemocytometers filled (certified Levy-Hausser chambers with Neubauer rulings), and duplicate counts were made.

Smears for the leucocyte differential and reticulocyte counts were made initially by both the slide and coverslip methods, but the latter is now used exclusively since a better distribution of cells is obtained. Two hundred cells were counted for the leucocyte differential. The smears for reticulocytes were prepared after staining with brilliant cresyl blue, and at least 500 red cells were counted. Occasionally the wet mount technique was employed. Wright's stain was used for the leucocyte smears and for counterstaining the reticulocyte

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smears. Cell volume was determined in duplicate with Wintrobe tubes.[17] Hemoglobin was determined by the Evelyn spectrophotometric method.[18]

Bone marrow samples were obtained immediately following sacrifice of the animals. Three different types of preparations were made: (1) Transverse sections were sawed from the upper third of the femur, and the marrow was shelled out of the bony ring, fixed in Zenker's formalin, sectioned, and stained with hematoxylin and eosin. (2) Impression imprints were made from femur marrow tissue and stained with Wright's or Giemsa's stain. (3) Marrow samples extruded from rib sections were diluted with heparin or heparinized serum, cover slips smears prepared, rapidly dried, and stained with Wright's or Giemsa's stain.[19] Differential counts of 500 or 1000 cells were made on the two latter types of preparations.

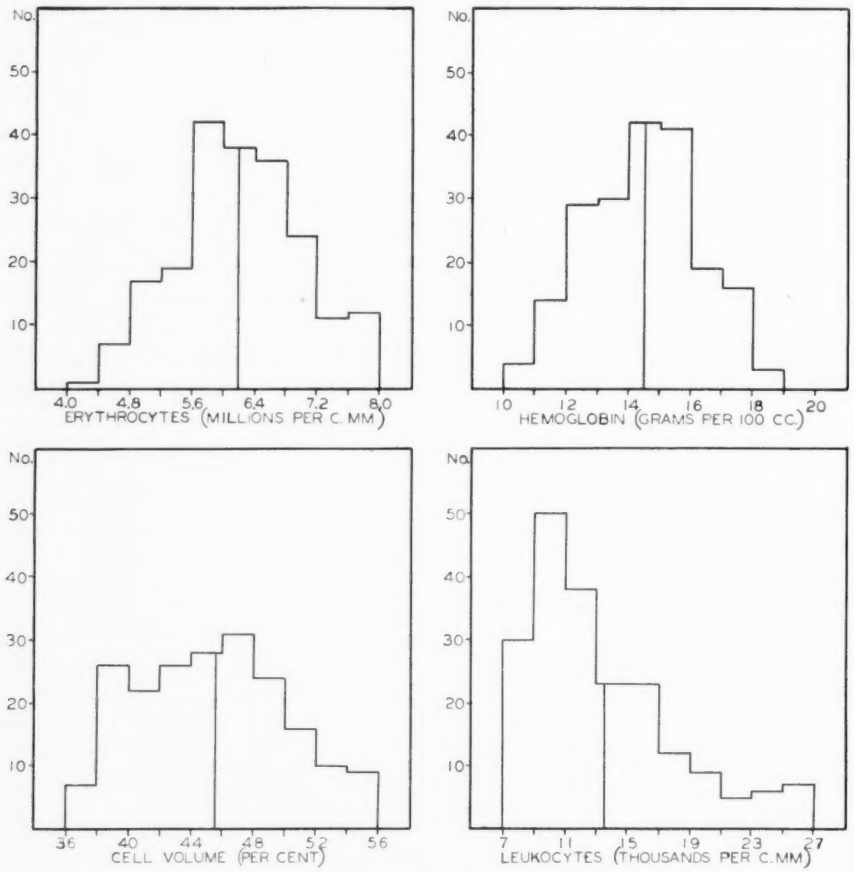

Fig. 1.—Frequency histograms of the hematology of 81 dogs.

RESULTS

Our observations on peripheral blood were made on 81 dogs and include 207 erythrocyte counts, 120 reticulocyte counts, 203 leucocyte counts, 199 determinations of cell volume, 198 determinations of hemoglobin, and 196 differential leucocyte counts. The observations on most individual animals were made during a period of one to two months; in 11, however, they extended over

a period of several months. The observations on the red and white blood cell counts, hemoglobin, and cell volume have been grouped in 2 ways, statistically analyzed, and are presented in Table I. All determinations on the 81 dogs are included in part A, and frequency histograms are shown in Fig. 1. Calculation from the mean values reveals a mean corpuscular volume of 73.4 cubic microns, a mean corpuscular hemoglobin of 23.7 micromicrograms, and a mean corpuscular hemoglobin concentration of 32.3 per cent. In part B the results of taking a single observation on each dog are shown; the last determination which had been made was arbitrarily chosen. These data are not significantly different, thus showing that an unequal number of determinations on some of the animals did not materially influence the analysis in part A. We noted that there was a general tendency for the erythrocyte, hemoglobin, and cell volume values to decrease slightly when the observations were extended over a long period. This trend was more definite in the first few weeks and was considered a result of training.

TABLE I

STATISTICAL ANALYSIS OF PERIPHERAL BLOOD HEMATOLOGY

		MEAN	STANDARD DEVIATION	PROBABLE ERROR OF MEAN	COEFFICIENT OF VARIATION	NO. OF OBSERVATIONS
A	Erythrocytes $\times 10^6$ per c.mm.	6.202	0.98	0.046	15.8	207
	Hemoglobin Gm. per 100 c.c.	14.56	2.05	0.098	14.1	198
	Cell Volume Per Cent	45.60	5.79	0.277	12.7	199
	Leucocytes $\times 10^3$ per c.mm.	13.523	4.48	0.212	33.1	203
B	Erythrocytes $\times 10^6$ per c.mm.	6.298	0.74	0.055	11.6	81
	Hemoglobin Gm. per 100 c.c.	14.65	2.27	0.170	15.5	81
	Cell Volume Per Cent	45.56	5.10	0.403	11.2	81
	Leucocytes $\times 10^3$ per c.mm.	13.110	4.43	0.336	33.8	81

TABLE II

COMPARISON OF MEAN VALUES REPORTED FOR HEMATOLOGY OF DOG BLOOD

NO. OF DOGS	HB	R.B.C.	CELL VOLUME	RETICULOCYTES	W.B.C.	DIFFERENTIAL					REF. NO.
						P	L	M	E	B	
	GM. %	PER CENT MM. $\times 10^6$	%	%	PER CENT MM. $\times 10^3$						
25	15.8	7.00	45.2								1
32	14.1	7.17	47.7		8.76						2
43-50	16.0	6.87	46.9								3
54	14.6	7.02	47.3								4
34	13.6	6.45	44.3	0.4	14.18						5
5-12	13.5	6.48		1.1	14.40						6
8-15		7.22			19.30	64.6	22.2	4.4	8.9		7
20		6.21			9.53	65.7	21.0	6.8	5.3		8
11-47	15.5	5.97		0-1.4	15.92	66.7	22.1	6.8	5.1		9
12		6.21			17.05	77.4	13.2	6.1	3.3		10
60	13.0	6.16	38.6	1.8	11.16	74.0	20.0	4.0	2.0		11
566	13.9	7.22			11.84	69.0	20.0	6.1	5.0	0.7	12
31	15.1	6.20			11.46	71.8	21.7	0.7	5.4		13
81	14.6	6.20	45.6	0-1.5	13.52	71.8	20.1	3.7	4.4	rare	*

*Mean values found by this laboratory.

The mean values of our data, compared with some of those reported in the literature, are shown in Table II. Our results are in general agreement with those of Bruner, et al.,[5] Mayerson,[11] Morris, et al.,[13] and others.

It was noted in making the reticulocyte count by both the wet and dry methods that there were many instances in which no reticulocytes were seen in counting 500 erythrocytes. Further examination of the smears, however, always revealed the presence of some reticulocytes.

We have observed, as others have reported, that no significant difference exists between the values obtained for males and females, for the different breeds, and that age apparently has little or no effect after the animals are 10 to 12 months old.

The mean and range of bone marrow differential counts on rib smears and femur imprints on the same eight dogs are shown in Table III. The impression imprints probably give a more nearly physiologic distribution of marrow cells. These preparations are difficult to prepare, however, do not stain well, and the counts obtained are somewhat selected, since usually only those cells on the periphery of each imprint can be differentiated. Smears from extruded rib marrow are easy to prepare, stain well, and cells over the entire smear are readily differentiated. It is our impression that representative marrow counts are obtained from these smears.

TABLE III

BONE MARROW DIFFERENTIAL COUNTS

TYPE OF CELL	RIB SMEARS		FEMUR IMPRINTS	
	MEAN	RANGE	MEAN	RANGE
Myeloblasts	0.6	0.2- 1.0	0.5	0.2- 1.0
Promyelocytes	1.6	0.7- 2.8	0.8	0.2- 1.1
Myelocytes	6.0	2.7-10.0	4.6	1.7- 8.0
Metamyelocytes	3.4	1.1- 4.6	3.3	2.0- 4.3
Bands, Neutrophilic	11.7	6.8-17.4	12.6	10.3-18.7
Segmenters, Neutrophilic	30.1	16.8-44.2	34.7	30.1-39.7
Eosinophils	2.0	0.4- 3.8	2.0	0.2- 3.3
Monocytes	0.2	0- 0.3		0- 0.1
Lymphocytes	0.9	0.2- 2.7	0.7	0- 3.4
Pronormoblasts	0.6	0.2- 1.2	0.3	0- 0.7
Normoblasts, Basophilic	7.8	6.4- 9.9	6.1	4.2- 8.4
Normoblasts, Polychromatic	16.4	11.0-26.0	16.5	13.3-19.8
Normoblasts, Orthochromatic	17.4	8.9-25.8	16.1	7.6-21.1
Megakaryocytes	0.5	0- 1.4	0.2	0- 0.3
Unclassified		0.8- 1.4		0.4- 3.1

Comparison of the data on rib smears with those on the femur imprints reveals a close similarity. Stasney and Higgins[15] have already pointed out that marrow from the rib, proximal femur, and middle femur show essentially the the same general trend in the relative percentage of distribution of cells, although the myeloid-erythroid ratio is somewhat higher in the rib. Our mean myeloid-erythroid ratio is slightly over one (range 0.6 to 2.4), thus agreeing with the data of Mulligan[16] and Alexandrov.[14] Stasney and Higgins found the myeloid-erythroid ratio consistently below one; however, their mean erythrocyte count was only slightly above 5.0 million. These observations lend further validity to the rib smear technique.

Histologic examination of the femur marrow sections revealed a wide variation in total cellularity. In general, active hematopoietic tissue was found around the periphery of the section and as small islands among the fat cells which filled the center of the section.

SUMMARY

A summary and statistical analysis of the data on the peripheral blood hematology of 81 dogs are presented. Differential cell counts of rib and femur marrow preparations from 8 dogs are included.

The authors are grateful to Dr. J. L. Schwind of the Department of Anatomy for many helpful suggestions.

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EFFECTS OF SULFAPYRAZINE AND SULFADIAZINE ON MICE INFECTED WITH HEMOLYTIC STREPTOCOCCUS, PNEUMOCOCCUS, AND STAPHYLOCOCCUS AUREUS*

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SUBSTITUTION of sulfanilamide in the sulfonamide group by replacement of one of the hydrogens by various heterocyclic compounds resulted in some cases in a substantial enhancement of therapeutic activity. The heterocyclic part of sulfadiazine, therapeutically a very active compound, consists of the pyrimidine ring where the 2 nuclear nitrogens are in the meta position to each other. Raiziss and Clemence[1] described a derivative containing a heterocyclic with 2 nuclear nitrogens in the para position. The same product was prepared independently by Elligson.[2] This derivative was obtained by the interaction of para-acetylaminobenzene sulfonyl chloride with 2-aminopyrazine. For the sake of brevity we named this product sulfapyrazine.

Sulfapyrazine has already been investigated clinically in the treatment of pneumonia and reported by Ruegsegger, Hamburger, Turk, Spies, and Blankenhorn.[3] The authors stated that 22 selected patients who received the drug by mouth showed prompt improvement and ultimate recovery with no significant signs of toxicity.

We are presenting studies on toxicity and blood levels and also on the therapeutic effect of sulfapyrazine in mice infected with various pathogenic organisms.

TOXICITY

Mice were fed a drug food diet containing ¼, ½, ¾, and 1 per cent of sulfapyrazine or sulfadiazine for a fourteen-day period. We have determined that mice consume on an average 4 Gm. per day of the drug diet. This would be equivalent to 0.010 Gm. of drug in ¼ per cent diet; 0.020 Gm. in ½ per cent; 0.030 Gm. in ¾ per cent; and 0.040 Gm. in 1 per cent. Table I shows that ¼ and ½ per cent drug diets containing either sulfapyrazine or sulfadiazine were well tolerated when fed for a period of fourteen days. Three-fourths per cent diet was tolerated only by 60 per cent of animals; 1 per cent diet of sulfapyrazine was tolerated by 50 per cent of animals at the end of fourteen-day period, while on a 1 per cent sulfadiazine drug diet all animals died after nine days of feeding.

BLOOD LEVEL DETERMINATIONS

Blood levels were determined by Bratton and Marshall's method.[4] From Table II it is apparent that sulfapyrazine on various drug diets produces com-

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TABLE I

CHRONIC TOXICITY FEEDING EXPERIMENTS DRUG DIET ADMINISTERED FOR 14 DAYS

COMPOUND	PER CENT DRUG WITH FOOD*	NO. MICE USED	PERCENTAGE OF SURVIVALS IN DAYS													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sulfapyrazine	¼ †	10	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	½	10	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	¾	10	100	100	100	90	90	90	90	90	90	70	60	60	60	60
	1	10	100	100	90	80	60	50	50	50	50	50	50	50	50	50
Sulfadiazine	¼	10	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	½	10	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	¾	10	100	100	100	100	100	90	90	90	90	70	70	70	60	60
	1	10	100	100	100	90	70	30	20	10	10	0				

*Mice consume on an average 4 Gm. per day of the drug diet.

†¼ per cent diet contains 0.010 Gm. of drug per day; ½ per cent, 0.020 Gm.; ¾ per cent, 0.030 Gm.; and 1 per cent, 0.040 Gm.

paratively high blood levels in mice ranging from 7.2 to 32 mg. per 100 c.c., which, however, are lower than those obtained with the respective sulfadiazine feedings.

COMPARATIVE THERAPEUTIC EFFECTS OF SULFAPYRAZINE AND SULFADIAZINE

In all of the therapeutic experiments drugs were administered to mice in food according to the method described by Litchfield, White, and Marshall.[5]

Mice were infected with the *Streptococcus hemolyticus* strain C203 using 1000 minimum lethal doses containing from 5 to 10,000 bacteria. According to Table III sulfapyrazine exerted a better therapeutic effect than sulfadiazine on mice with streptococcus infection fed the ¼ per cent drug diet. On the ½ per cent drug diet sulfadiazine gave better results than sulfapyrazine. Under the same condition, sulfanilamide proved to be less effective.

TABLE II

AVERAGE OF DAILY RECORDED BLOOD LEVELS IN MICE FED DRUG DIET FOR 10 DAYS

PER CENT DRUG IN FOOD	BLOOD CONCENTRATIONS SULFAPYRAZINE MG. PER 100 C.C.*	FREE DRUG SULFADIAZINE MG. PER 100 C.C.*
¼	7.2	15
½	10.4	26
¾	28	38
1	32	43

*The averages represent tests using from 4 to 6 mice.

TABLE III

COMPARATIVE THERAPEUTIC EFFECT OF SULFAPYRAZINE AND SULFADIAZINE DRUG ADMINISTERED WITH FOOD FOR TEN DAYS

STREPTOCOCCUS HEMOLYTICUS INFECTION, STRAIN C203

PER CENT DRUG WITH FOOD	DRUG	NO. OF MICE USED	PERCENTAGE OF SURVIVALS DAYS 1	2	3	4	5	6	7	14	21	28
¼	Sulfapyrazine	25	100	100	100	100	100	100	100	96	96	96
	Sulfadiazine	25	96	92	92	92	92	92	92	88	84	84
½	Sulfapyrazine	80	91	91	82	80	76	75	70	59	59	50
	Sulfadiazine	80	100	98	84	84	81	78	78	74	71	71
	Sulfanilamide	25	84	84	68	60	60	60	60	56	56	56
	Controls	27	4	0								

Heart cultures of autopsied mice who died before the 28th day invariably were positive for Streptococci.

In the experiments on Type I pneumococcus infection we used strain M-36.[6] Mice were infected with 10 minimum lethal doses, the total content of inoculum being about 100 bacteria. This strain proved to be of high virulence. As seen from Table IV, 88 per cent of mice on the ¼ per cent sulfapyrazine diet survived seven days. After fourteen days the survival rate dropped to fifty per cent. On ¼ per cent sulfadiazine diet, 33 per cent of mice survived seven days and after fourteen days 23 per cent, indicating a better effect for sulfapyrazine. On a ½ per cent drug diet sulfadiazine was somewhat less effective than sulfapyrazine.

In our experiments on Type II pneumococcus infection, we used strain M-48 as described in previous publication.[6] Mice were infected intraperitoneally with 10 minimum lethal doses containing about 250 bacteria.

From Table IV one can see that mice fed ¼ per cent of sulfapyrazine diet showed 96 per cent survivals after four days, 44 per cent after fourteen days and 40 per cent after twenty-eight days of observation.

Under the same conditions, sulfadiazine therapy resulted in 33 per cent survivals after four days and only 5 per cent survivals after fourteen and twenty-eight days. On ½ per cent diets both drugs after twenty-eight days gave equally good therapeutic results.

Pneumococcus Type III proved to be more resistant to drugs than Type II. We used 10 minimum lethal doses which contained about 200 bacteria of strain M-50. With a ¼ per cent diet, 20 per cent of the mice fed sulfapyrazine survived three days, while at the end of twenty-eight days 5 per cent survived. Mice given ¼ per cent sulfadiazine diet showed about the same results with a survival rate of 20 per cent after three days and 7 per cent at the end of twenty-eight day period.

TABLE IV

THERAPEUTIC EFFECTS OF SULFAPYRAZINE AND SULFADIAZINE IN MICE DRUG ADMINISTERED WITH FOOD FOR TEN DAYS

PNEUMOCOCCUS	PER CENT DRUG WITH FOOD	COMPOUND	NO. MICE USED	PERCENTAGE OF SURVIVALS DAYS									
				1	2	3	4	5	6	7	14	21	28
Type I Strain M-36	¼	Sulfapyrazine	40	98	98	95	88	88	88	88	50	50	50
	½	Sulfapyrazine	40	98	85	85	85	85	83	83	73	70	70
	¼	Sulfadiazine	40	100	55	38	35	33	33	33	23	23	23
	½	Sulfadiazine	40	98	95	95	95	93	93	93	63	55	55
		Controls	18	2	0								
Type II Strain M-48	¼	Sulfapyrazine	50	96	96	96	96	90	90	86	44	40	40
	½	Sulfapyrazine	40	100	100	93	93	90	88	85	60	55	55
	¼	Sulfadiazine	40	95	75	50	33	25	25	15	5	5	5
	½	Sulfadiazine	40	100	100	100	100	93	93	88	58	55	55
		Controls	23	4	0								
Type III Strain M-50	¼	Sulfapyrazine	20	95	35	20	10	10	10	10	10	5	5
	½	Sulfapyrazine	40	100	90	70	65	58	58	55	40	38	38
	¼	Sulfadiazine	15	87	33	20	13	7	7	7	7	7	7
	½	Sulfadiazine	40	100	85	55	50	43	33	30	28	25	25
		Controls	17	18	0								

Heart cultures of autopsied animals invariably were positive for Pneumococci.

On the ½ per cent diet at the end of three days sulfapyrazine had 70 per cent, while sulfadiazine had only 55 per cent survivals. At the end of twenty-eight days, 38 per cent survivals were observed with sulfapyrazine and only 25 per cent with sulfadiazine. In this case sulfapyrazine showed better therapeutic effect.

Staphylococcus aureus infection was produced in mice by the intravenous administration (saphenous vein) of 0.2 c.c. of a saline suspension containing about 8 billion bacteria. This culture had five passages through mice prior to its use. As seen from Table V, 50 per cent of control mice died at the end of seventy-two hours and the rest died seven days after infection.

On a ¼ per cent sulfapyrazine diet, 40 per cent of the animals survived for eleven days and 30 per cent survived fourteen days, showing a slightly better effect than sulfadiazine.

On a ½ per cent drug diet, sulfadiazine proved to be better.

TABLE V

COMPARATIVE THERAPEUTIC EFFECTS OF SULFAPYRAZINE AND SULFADIAZINE DRUG ADMINISTERED WITH FOOD FOR TEN DAYS

STAPHYLOCOCCUS AUREUS INFECTION, STRAIN 209

PER CENT DRUG WITH FOOD	DRUG	NO. MICE USED	PERCENTAGE OF SURVIVALS DAYS													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
¼	Sulfapyrazine	10	100	100	100	90	80	80	80	80	70	60	40	40	30	30
	Sulfadiazine	10	100	100	90	90	80	80	60	50	50	40	30	20	20	20
½	Sulfapyrazine	10	100	100	90	70	60	60	30	30	30	20	0			
	Sulfadiazine	10	100	100	90	90	80	80	80	80	80	70	70	60	50	40
	Controls	10	100	70	50	30	20	10	0							

Cultures of kidneys of autopsied animals were positive for *Staphylococcus aureus*.

DISCUSSION

(a) Sulfapyrazine, an isomer of sulfadiazine, by this and further investigations may prove to possess certain advantages over sulfadiazine in clinical application and, therefore, may be valuable.

(b) We have established that blood levels of mice kept on drug diets containing sulfapyrazine are generally lower than those of sulfadiazine, being higher than blood levels obtained with other sulfanilamide derivatives.

(c) This report is the first, according to our knowledge, of an extensive study of sulfapyrazine by the food drug diet method on various types of pneumococcus.

(d) By a careful series of experiments, we established that sulfapyrazine has either equal or somewhat better therapeutic effect on food drug diets at lower blood levels than sulfadiazine.

CONCLUSIONS

1. Mice tolerate sulfapyrazine and sulfadiazine on a ¼ and ½ per cent food diet equally well, while a ¾ and 1 per cent drug diet sulfapyrazine is somewhat better tolerated than sulfadiazine.

2. Blood levels on ¼ and 1 per cent drug diets ranged from 7.2 to 32 mg. per 100 c.c.

3. In beta-hemolytic streptococcic infection both drugs were of equal therapeutic effectiveness.

4. In Type I Pneumococcus infection, mice fed on ¼ and ½ per cent sulfapyrazine drug diets showed a better survival rate.

5. In Type II pneumococcus infection, sulfapyrazine showed a greater survival rate, than sulfadiazine on ¼ per cent and equal on ½ per cent drug diets.

6. In Type III pneumococcus infection both drugs were equally effective on the ¼ per cent diet, while sulfapyrazine gave better results on a ½ per cent diet.

7. According to our experiments, in *Staphylococcus aureus* infection, sulfapyrazine and sulfadiazine proved to be equally effective.

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A NEW METHOD FOR THE EVALUATION OF DRUGS AFFECTING THE REACTION OF MICE TO PAIN STIMULATION*

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IN recent years a number of new methods for the testing of analgesic drugs in animals has been reported. Without claim for completeness we mention here the methods of Macht and Macht,[1] d'Amour and Smith,[2] and of Andrews and Workman,[3] the last two methods being modifications of the principle developed by Hardy, Wolff, and Goodell.[4] In our experience, results can be obtained with all of them. We found that there was little consistency, however, inasmuch as certain drugs were found relatively more potent with one of the methods than with another. On account of this we employ as a rule several procedures in estimating analgesic properties.

The necessity of immobilizing the animal during the test represents, in our opinion, a common disadvantage of these methods. Since it is not certain whether any method of determining analgesia in animals measures "pain" in the sense of the human quality or just a reflex, we believe the experimental conditions should enable the animal to be as unrestricted in its movements as possible.

This demand is satisfied in a procedure used by Straub and Triendl[5] and based upon a method developed by Forst,[6] which consists essentially of having a mouse move about freely on a disc and measuring the movements by means of a work adder; a clamp is then applied to the nose or rectum and the reaction is measured by the increased activity of the animal. We found it profitable to substitute the continuous stimulation caused by the clamp by an intermittent stimulation which can also be easily varied in degree. Our procedure does not require complicated or expensive apparatus and has proved valuable in our hands in combination with other methods.

METHOD

Mice are used for this test. The animals are stimulated through their feet in a basket made of plastecele and aluminum with a bottom made of lightweight

*From the Department of Pharmacological Research, Abbott Laboratories.
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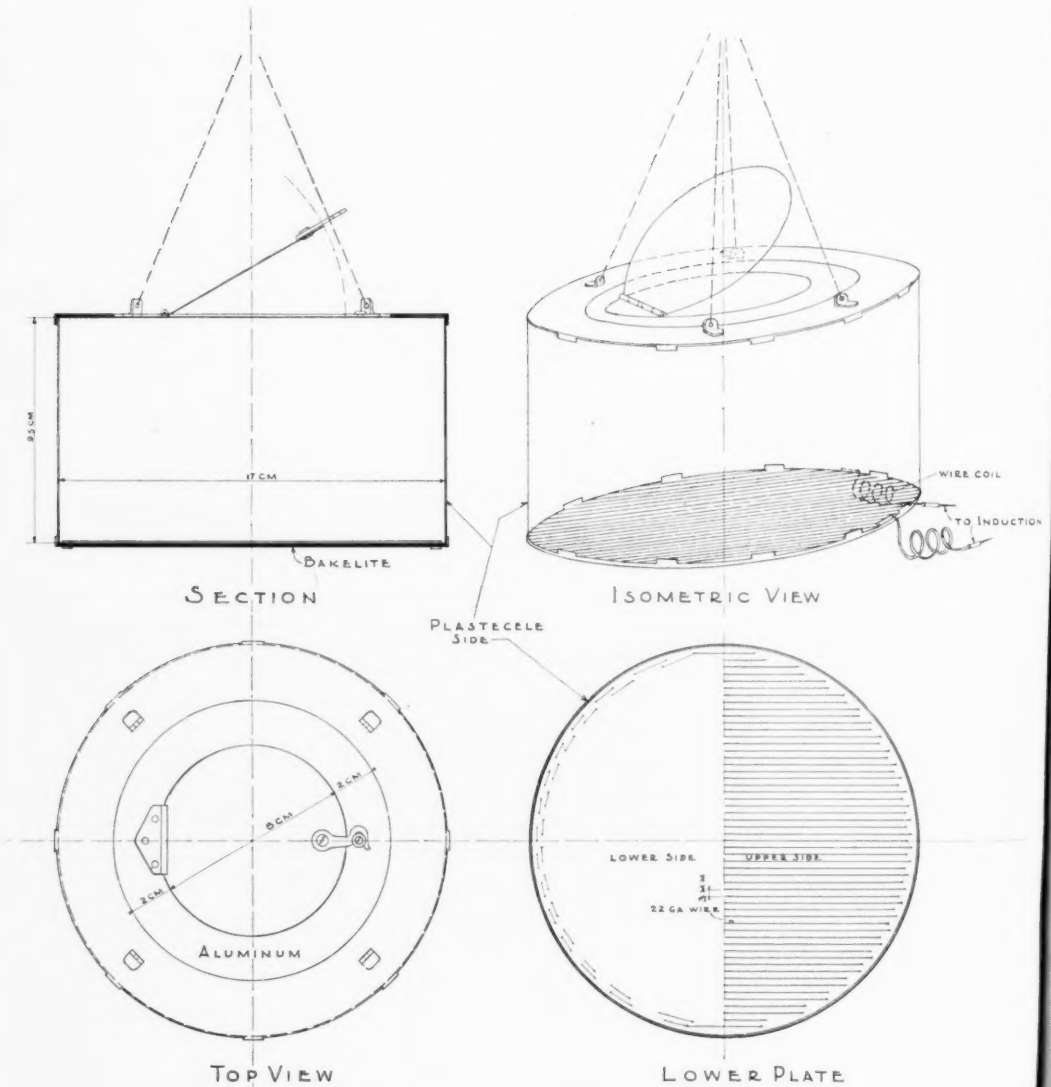
8.5CM
17CM
BAKELITE
SECTION
WIRE COIL
TO INDUCTION
ISOMETRIC VIEW
PLASTECELE SIDE
2CM
6CM
2CM
ALUMINUM
TOP VIEW
LOWER SIDE
UPPER SIDE
22 GA WIRE
LOWER PLATE
STIMULATION BASKET

Fig. 1.

bakelite. The basket resembles a small hatbox. It is 17 cm. in diameter and 9.5 cm. in height. The bottom of the basket is covered with rows of fine copper wire about 3.0 mm. apart. These wires are strung across the bottom so that one wire is positive and the adjacent one negative. This is accomplished by stringing one continuous wire in rows about 6 mm. apart and then stringing another wire in between the rows of the first strung wires. Care must be taken so that the two wires are well insulated from each other. (Fig. 1 shows how this is accomplished.) The basket is suspended from a bellows made by stretching a condom over a large-sized heart chamber. The basket hangs by means of strings and a wire hook made fast in a small, square cardboard and fastened to the condom with rubber cement. The heart chamber is connected to a tambour and writing arm with rubber tubing. The tambour is held in place by a clamp and ring stand. Any movement in the basket sends air impulses through the tubing to the tambour and arm. Movements of the animal are recorded on a slowly moving kymograph. The basket is connected to the secondary coil of a Harvard inductorium, and the primary (4 volts) current is made intermittent by means of a Harvard electric time clock or metronome. The physical considerations of the Harvard inductorium are explained in detail by Macht and Macht.[1] (Fig. 2 shows the complete apparatus.)

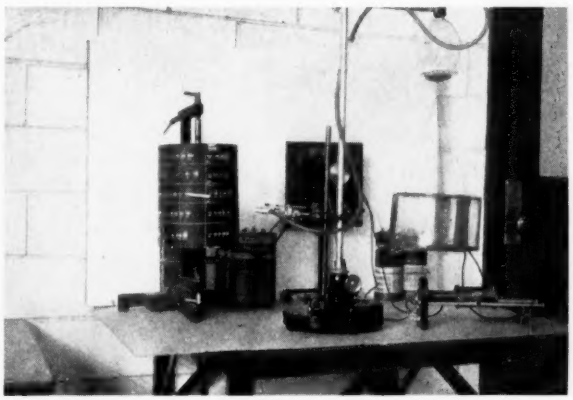

Fig. 2.

At least two mice are run on a dose level. The animals are first tested as normal mice. A tracing is always made with no stimulation, covering about one inch of paper. Then, the secondary coil is set at 7 cm., and the time clock is set in motion. A short tracing is made and the current is shut off. The secondary coil is then moved to 6.75 cm. A tracing is made, the current interrupted, the coil shifted to 6.5 cm., and so on, in 0.25 to 0.5 cm. intervals. As the stimulus becomes stronger, the mice start to make jumping movements which increase to a maximum, usually at about 5.5 to 4.0 cm. secondary coil distance. There is a considerable variation in the sensitivities of the individual animals that has to be taken into consideration for the evaluation of an analgesic. Mice which fail to show an increment of reaction with increased stimulation or are too restless are discarded.

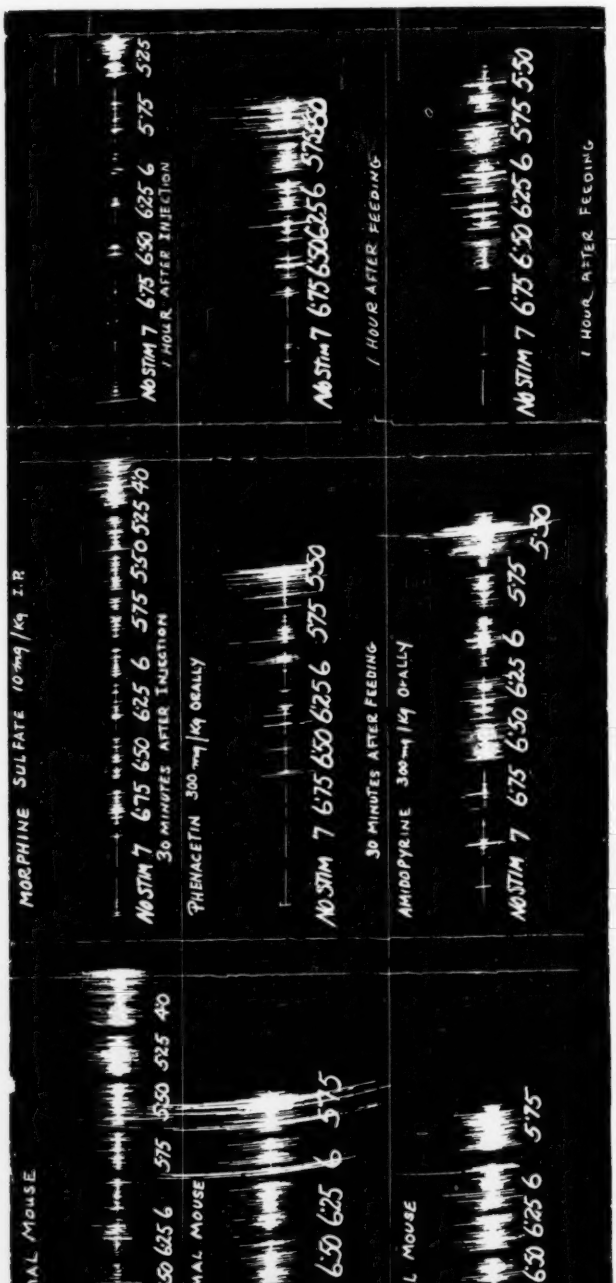
NORMAL MOUSE
MORPHINE SULFATE 10 mg/Kg I.R
NO STIM 7 6.75 6.50 6.25 6 5.75 5.50 5.25 4.0
NORMAL MOUSE
NO STIM 7 6.75 6.50 6.25 6 5.75
NORMAL MOUSE
NO STIM 7 6.75 6.50 6.25 6 5.75
30 MINUTES AFTER INJECTION
NO STIM 7 6.75 6.50 6.25 6 5.75 5.50 5.25 4.0
PHENACETIN 300 mg/Kg ORALLY
NO STIM 7 6.75 6.50 6.25 6 5.75 5.50
30 MINUTES AFTER FEEDING
AMIDOPYRINE 300 mg/Kg ORALLY
NO STIM 7 6.75 6.50 6.25 6 5.75 5.50
1 HOUR AFTER INJECTION
NO STIM 7 6.75 6.50 6.25 6 5.75 5.25
1 HOUR AFTER FEEDING
NO STIM 7 6.75 6.50 6.25 6 5.75
1 HOUR AFTER FEEDING
NO STIM 7 6.75 6.50 6.25 6 5.75 5.50

FIG. 8.

TABLE I

EFFECT OF ORAL ADMINISTRATION OF ANALGESICS ON THE PAIN THRESHOLD

MOUSE NO.	DRUG ADMINISTERED	DOSE MG./KG.	PAIN THRESHOLD		
			NORMAL	15 TO 30 MIN. AFTER DOSE	1 TO 2 HOURS AFTER DOSE
			CM.	CM.	CM.
1	Phenacetin	300	6.75	6.75	6.0
2	Phenacetin	750	6.5	6.5	6.0
3	Phenacetin	750	7.0	6.0	5.5
4	Phenacetin	1000	7.0	5.0	4.0
5	Amidopyrine	250	7.0	5.5	6.0
6	Amidopyrine	300	7.0	6.0	5.5
7	Aspirin	500	6.5	6.5	6.5
8	Nembutal	55	7.0	7.0	6.5
9	Nembutal	75	7.0	4.0	4.0
10	Nembutal*	110	7.0	4.0	0.0

*Animal was in surgical anesthesia.

TABLE II

EFFECT OF INTRAPERITONEAL INJECTION OF MORPHINE ON THE PAIN THRESHOLD

MOUSE NO.	DRUG ADMINISTERED	DOSE MG./KG.	PAIN THRESHOLD		
			NORMAL	15 TO 30 MIN. AFTER DOSE	1 TO 2 HOURS AFTER DOSE
			CM.	CM.	CM.
1	Morphine SO_4	4	7	6.5	6.5
2	Morphine SO_4	6	7	6.0	6.0
3	Morphine SO_4	6	7	6.0	6.0
4	Morphine SO_4	10	7	5.5	6.0
5	Morphine SO_4	10	7	5.25	5.25
6	Morphine SO_4	10	7	6.5	5.0
7	Morphine SO_4	10	7	5.0	4.5
8	Morphine SO_4	10	6.5	5.5	4.5
9	Morphine SO_4	15	7	5.5	5.5
10	Morphine SO_4	20	7	5.5	5.5

After the normal reactions of the mouse have been recorded, the drug under investigation is administered either by mouth or by intraperitoneal injection. For the first test we give usually 25 to 50 per cent of the L.D.50. Later the dose has to be adjusted according to experience. Tests are made in intervals of 20, 30, or 60 minutes depending on the type of drug and method of administration. Fig. 3 shows tracings of such experiments, and a summary of the effects of a few well-known analgesics administered orally is given in Table I; the columns headed "Centimeter" show the reading of the secondary coil at which a definite stimulation was recorded after the drug. Necessarily, this criterion is somewhat subjective with the individual investigator, but if interpretations are always made on the same basis, the results become comparable. Table II shows the effects of morphine by intraperitoneal injections. Whereas the sensitivity of this method compares favorably with other procedures, we failed to obtain a definite analgesic effect with aspirin after oral administration with the chosen dose, but phenacetin and amidopyrine exhibited definite analgesic action. Nembutal was ineffective at 55 mg./kg., a dose not producing marked depression. Analgesia appeared with the definitely depressing dose of 75 mg./kg. and became complete with 110 mg./kg. In this stage no reaction was elicited. This proves that our method of stimulation produces central effects and not merely peripheral con-

traction by means of peripheral stimulation. Newer drugs which were tested and found analgesic by this procedure have proved to be effective in preliminary clinical investigations.

We are aware that, in testing of analgesic drugs, not only their central true analgesic action but also other effects have to be taken into consideration. Thus, morphine possesses a definite stimulating effect upon the spinal centers, an action which results in the well-known Straub phenomenon; however, in our test, signs of the spinal stimulation do not show up (see Fig. 3, upper tracings). The analgesic effect of aspirin, so conspicuous in humans, appears to be difficult to demonstrate in animals. This has been substantiated recently again by Smith, D'Amour, and D'Amour.[7] The barbiturates which exert a general cerebral depressing effect fail to influence selectively the pain centers, as is demonstrated by our failure to obtain analgesic effects with sedative doses; this is in agreement with clinical experience. Taking all these factors in consideration, one has to recognize the limitations of new methods endeavoring to measure a particular property in the presence of other factors which will influence a test.

We believe that the above described method will be of help to investigators in this field in conjunction with other procedures. The absence of restraint exerted on the animals, the recording of the reactions of the animals, and the possibility of using large numbers of small animals have definite advantages. The apparatus needs little care, but the basket should be inspected frequently and urine and feces should be removed to prevent short circuit. The air transmission system must of course be leakproof and should be adjusted to optimal sensitivity.

SUMMARY

A new method for testing analgesic drugs in mice is described. The method permits the application of an intermittent and increasing stimulus to mice without restriction of their movements and obtains direct records of their reactions.

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CHRONIC INDURATIVE PNEUMONIA RESULTING FROM CARDIOSPASM*

A Case With Nonpathogenic Acid-fast Bacilli in the Sputum

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PNEUMONITIS complicating cardiospasm does not appear to be a very common condition. Isolated cases have been reported.[1, 2] On the other hand, Sturtevant,[3] in an article reviewing the literature on cardiospasm, fails to mention pulmonary complications, apparently because of their rarity. Even more uncommon have been reports of the isolation of nonpathogenic acid-fast organisms from the sputum in this condition. Baldwin[4] has recently reported such a case, and a similar one has been described by Cummins and Williams.[5] These two cases are apparently the only ones to be found in the literature in which nonpathogenic acid-fast bacilli have been found in the sputum in this condition. A third case has lately come under our observation.

CASE HISTORY

G. H., a white female, single, aged 65 years, a bookkeeper, was admitted to the Laurel Heights State Tuberculosis Sanatorium on December 8, 1941. There was no history of exposure to tuberculosis.

Past History.—In 1922 the patient began to have difficulty in swallowing, with regurgitation of small amounts of food, but not of whole meals. There was no history of having swallowed any irritants. At that time she manifested mild jaundice, and her gall bladder, containing stones, was removed. Further studies while she was in the hospital revealed moderate cardiospasm. No dilatation was then felt to be necessary.

During the past twenty years she has been troubled by this regurgitation for periods varying in length from a few hours to three days, and then would be entirely free from this symptom for intervals up to several months. She denied aspirating vomitus at any time.

Present Illness.—Gradually, over the past year and a half, there has been a loss of 16 pounds in weight. This was probably because she ate only small amounts of food in an effort to ward off regurgitation. Seven weeks before admission the patient developed weakness, fever, and night sweats. She also began to cough, and she raised some mucoid sputum which was not foul but did contain a few food particles. On bed rest at home she failed to improve and came to Laurel Heights on December 8, 1941.

On admission her general condition was poor. Her best previous weight had been 136 pounds, but she was now down to 87. Stereoroentgenograms of the chest, taken on December 9, 1941, showed mottled densities throughout the lung field on the right, with consolidation from the level of the third to the seventh vertebral spines. On the left, there were conglomerate patches in the lower lobe (Fig. 1.). Physical examination was noncontributory, except for the lungs. There were signs of infiltration, with medium and fine râles, over the areas of disease seen in the x-ray.

The patient had asymptomatic *Bacillus coli* bacilluria, with white blood cells, but no other urinary abnormalities. Hemoglobin was 11.5 Gm. (74 per cent); red blood cells, 3.85; white blood cells, 16,500, with polymorphonuclear cells 85 per cent, lymphocytes 9 per cent,

*From the Laurel Heights State Tuberculosis Sanatorium.
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monocytes 5 per cent, and eosinophils 1 per cent. Sedimentation rate (Cutler) was 27.0 and 28.5 mm. Kline diagnostic test was negative. A tuberculin test was highly positive to the second strength of purified protein derivative.

X-rays of the stomach and intestines were negative. Barium was retained in the esophagus, however, for as long as ten hours, revealing obstruction at the cardiac orifice. The lower third of the esophagus was mildly and uniformly dilated. No diverticulae could be seen (Fig. 2).

The patient was felt to have a cardiospasm of twenty years' duration. By unconsciously aspirating food, probably during sleep, she had developed a chronic, bilateral induration pneumonia.

Two specimens of sputum, raised from the lungs, contained large numbers of acid-fast bacilli. Morphologically these were very similar to tubercle bacilli. The working diagnosis of aspiration pneumonia led to further studies which demonstrated the organisms to be nonpathogenic, acid-fast saprophytes.

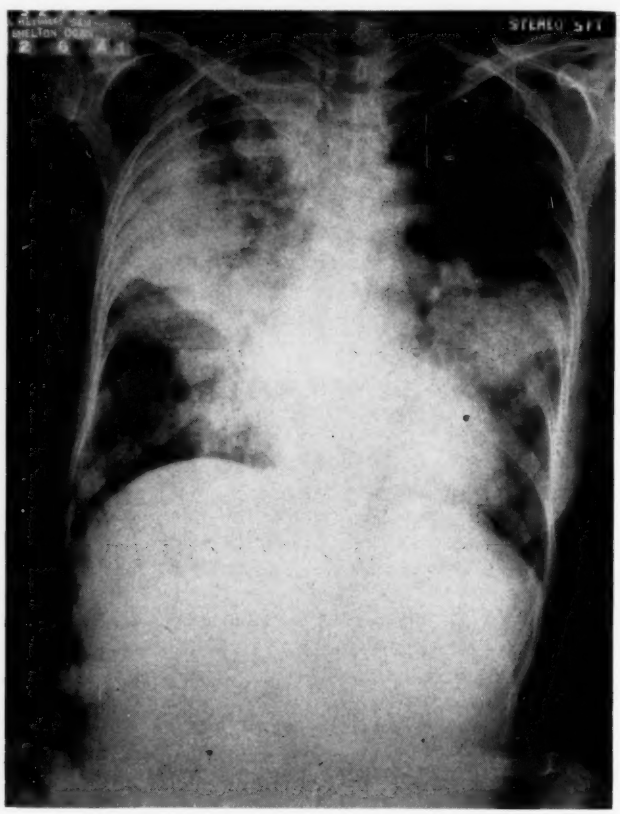

Fig. 1.—X-ray of the chest on admission 12/8/41.

On bed rest the patient ran a continuous fever from 100° to 101° F. Her pulse ranged from 75 to 85. During her two months' stay in the Sanatorium there was no significant change in the appearance of serial x-rays of the lungs. Occasional regurgitation persisted, but she gained a little in weight and strength. On February 12, 1942, she was discharged, to receive treatment elsewhere for the cardiospasm.

In March, 1942, Miss H. was treated by Doctor Norton Canfield at the New Haven Hospital. Dilatation of the lower end of the esophagus was accomplished by means of the McKenzie dilator. After two weeks, good esophageal function was obtained. Since that time she has been able to swallow and retain both liquids and solid foods without difficulty.

The patient returned to our Out-patient Department for a check-up on October 9, 1942. Her general condition was much improved and she had gained about twenty pounds. She felt

stronger, her fever had disappeared, and she had practically no cough or expectoration. Chest roentgenograms showed pronounced clearing of the densities in both lungs, although a good deal of chronic, fibrous infiltration remained (Fig. 3). No acid-fast organisms could be demonstrated in the sputum by smear or culture at this visit.

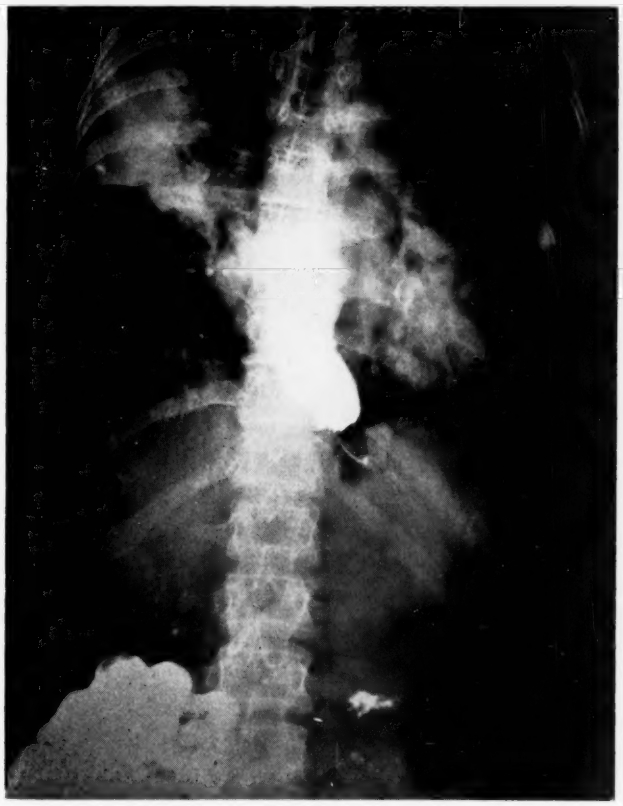

Fig. 2.—X-ray taken 10 hours after barium meal showing cardiospasm.

THE ORGANISM

On admission, in December, 1941, acid-fast rods were found in good numbers in the sputum on direct smear. Morphologically they closely resembled tubercle bacilli, although a few acid-fast coccoid bodies were also present. The organism grew well in Hamburg broth in twenty-four hours. On dextrose agar, after forty-eight hours, the colonies were smooth and had a slight cream color, taking on an orange tinge as the culture aged. No carbohydrates were fermented. After several subcultures the morphology changed markedly. The rods disappeared, leaving mostly acid-resistant, deep-staining, coccoid bodies, with some fine, faintly staining filamentous forms. Guinea pigs inoculated with the bacteria did not develop tuberculosis, nor did they become sensitized to old tuberculin.

Doctor Friend Lee Mickle of the Connecticut State Laboratory and Mr. William Steenkin, Jr., at the Research and Clinical Laboratory of Trudeau, New York, studied these cultures with us. It was agreed that the organism was a nonpathogenic acid-fast saprophyte.

Doctor Edward R. Baldwin of Saranac Lake, New York, made a tuberculin from cultures of this saprophyte. Skin-tested with 0.1 mg. of this autogenous tuberculin, the patient reacted with an area of erythema measuring 3.5 × 4.0 cm. and marked induration. An intradermal control test, using 0.1 mg. of old tuberculin, produced an area of erythema 4.5 × 6.5 cm. with pronounced induration.

Twelve other patients with proved, active pulmonary tuberculosis were skin-tested, using 0.1 mg. of the autogenous tuberculin in six patients and 1.0 mg. in the remaining six. Only two patients, both of whom received the 1.0 mg. dose of the autogenous tuberculin, showed a mild, but typical positive reaction, with erythema and induration. All twelve cases reacted strongly to 0.1 mg. of regular old tuberculin.

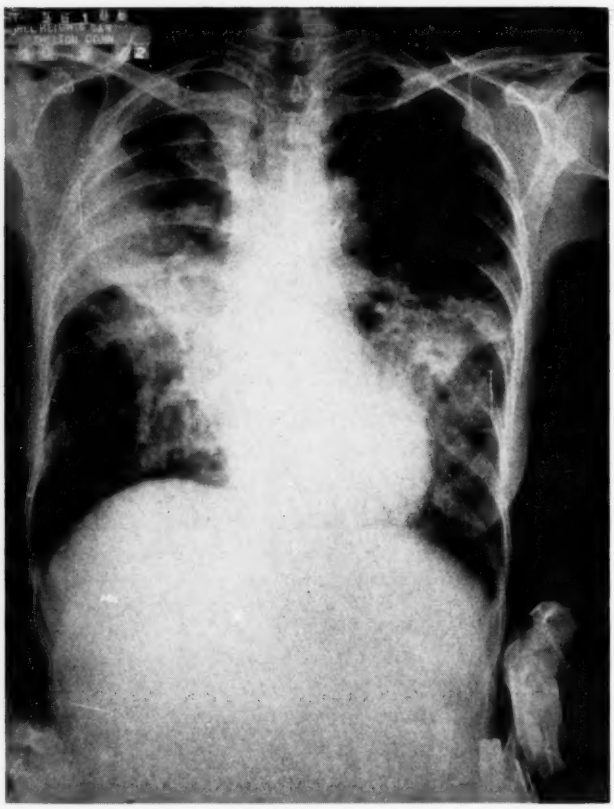

Fig. 3.—X-ray of the chest taken on 10/9/42, seven months after treatment of cardiospasm by dilatation. Moderate clearing of infiltration in both lungs.

These studies showed that the patient had acquired a marked degree of sensitivity to the nonpathogen in her sputum. Almost complete lack of reaction in the tuberculous controls demonstrated that this could not be explained adequately by cross sensitivity.

COMMENT

This report of another case of aspiration pneumonitis, secondary to cardiospasm, in which acid-fast bacilli have been found in the sputum, would suggest

a relationship that is more than accidental. As proposed by Baldwin,[4] a logical explanation for the source of the nonpathogenic acid-fast bacilli is to be found in the aspiration of food particles from the esophagus into the lungs. There the organism may become established and occasionally make its appearance in the sputum. Similar nonpathogenic acid-fast organisms have been cultured from many raw fruits and vegetables.[4]

The question arises whether or not this acid-fast saprophyte had developed some slight degree of pathogenicity for this particular patient. Probably not, since it did not seem to influence the course of the aspiration pneumonia and did not produce disease in guinea pigs. On the other hand, it appears that the bacteria did not merely lodge inertly in the bronchial tree, but established themselves as facultative parasites. This assumption is supported by the development of the marked sensitivity, absent in controls, to the tuberculin prepared from the acid-fast bacilli in her sputum.

The finding of these acid-fast rods in the sputum on direct smear is of definite interest. The differentiation between them and tubercle bacilli on the basis of their morphologic characteristics would be extremely difficult. Thorough bacteriologic studies should be performed on any patient suspected of having an aspiration pneumonitis, in which acid-fast organisms are found in the sputum. Otherwise a mistaken diagnosis of pulmonary tuberculosis may readily be made.

The short length of time during which the acid-fast bacilli were found in the sputum and the rapidity with which they changed to coccoid forms may explain why more of these cases have not been reported. Apparently only under certain favorable cultural environments do they retain their acid-fast bacillary form. This is true both in vivo and in vitro. The rapidity with which the organism multiplies (twenty-four to forty-eight hours) on most laboratory media is one means of differentiating them from tubercle bacilli.

SUMMARY

A case of aspiration pneumonitis resulting from cardiospasm is reported, in which saprophytic acid-fast bacilli were found by direct smear in the sputum. Morphologically these bacteria resembled tubercle bacilli. The bacteriologic characteristics of the organism are described.

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CLINICAL CHEMISTRY

STUDIES IN PROTHROMBIN: V. ARTERIAL AND VENOUS PLASMA PROTHROMBIN TIME IN MAN*

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THE liver is essential for the elaboration of prothrombin.[1] Although other tissues seem not to be involved directly in the process, nevertheless, recent observations suggest that under certain conditions additional mechanisms might contribute toward the maintenance of prothrombin at a level (or activity) higher than normal. Thus, hyperprothrombinemia has been demonstrated in (a) acute thrombophlebitis and the initial stage of thrombo-embolization[2, 7] and (b) multiple myeloma.[3]

It has been noted by Andrus, Lord, and Kauer[4] in 17 out of 20 experiments on dogs that blood obtained from the left ventricle exhibited less prothrombin activity than that removed from the right ventricle. The average difference was 10.6 per cent, determined by the two-stage method of Warner, Brinkhous, and Smith.[5] The visceral and peripheral arterial and venous bloods showed no significant difference.

These observations led to the present investigation to determine what is the relationship between the peripheral arterial and venous blood plasma prothrombin time in man.

The prothrombin times of arterial and venous plasma of twenty-six patients were studied. In 11 of these, the venous plasma prothrombin times were normal; in the remaining 15, the prothrombin times were prolonged as a result of existing liver disease, nutritional (vitamin K?) deficiency, or therapeutic agents which decrease the level (or activity) of prothrombin (salicylates, sulfanyl compounds, Dicumarol).

Method of Prothrombin Assay.—The procedure used to estimate the prothrombin time is based upon the single-stage method of Quick[6] and includes estimation of the prothrombin time of whole and diluted (12.5 per cent) plasma. The rationale, clinical and experimental applications have been set forth in previous communications.[2, 3, 7-11] The greater sensitivity of 12.5 per cent plasma over that of whole plasma also has been demonstrated.[12, 13]

Thromboplastin prepared from fresh rabbit lung was used. The established normal standard for 12.5 per cent (venous) plasma was 39.5 seconds (Standard deviation = ±2.5). All estimations were done in duplicate.

The specimens of venous blood were obtained in the usual manner of venipuncture. The arterial blood samples were taken from the radial artery. The cutaneous area of puncture was first anesthetized with 1 per cent novocain

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Plasma was obtained by the addition of 4.5 c.c. of blood to 0.5 c.c. 1/10 molar sodium oxalate and centrifugation.

Results.—More than 100 estimations of the prothrombin time of arterial and venous plasma obtained from the 26 individuals were made. In three patients serial estimations were made daily for one week. In every instance the difference between the arterial and venous plasma prothrombin time was found to be within the limits of statistical variation. In three cases the differences were on the first examination slightly in excess of these limits. Repetition of the determinations on subsequent days, however, revealed no significant difference between the respective arterial and venous plasma prothrombin time.

Discussion.—In normal individuals the prothrombin time of arterial blood was a trifle greater than that of venous blood. The magnitude of the difference was in almost every instance less than three seconds and hence within normal variation and consequently to be considered as statistically not significant.

The arterial and venous plasma prothrombin time in four cases of Laennec's cirrhosis, although moderately or markedly prolonged,* were almost identical in each instance.

Prothrombinopenia, whether induced by agents such as Dicumarol,[2, 11] salicylate[12, 13] or sulfanyl compounds,[15, 16] or occurring spontaneously as a result of chronic debility (nutritional [vitamin K?] deficiency), does not alter the relationship which appears normally to exist between the prothrombin time of arterial and venous plasma.

It is apparent, therefore, that in man (as in dogs[4]) no significant difference between arterial and venous plasma prothrombin time is demonstrable. Should such a variation arise it would be distinctly abnormal. It might conceivably occur as a result of an unusual interchange within the pulmonary system or at the peripheral capillaries.

It is here established that for practical purposes arterial or venous blood may be used interchangeably for estimation of the prothrombin time.

Summary.—The greater sensitivity of diluted plasma as compared with whole plasma for estimation of prothrombin time is emphasized.

The prothrombin time of peripheral arterial plasma is generally a trifle in excess of that of venous plasma. The difference, however, is within the range of statistical variation and consequently not significant.

The findings herein recorded indicate that when prothrombinopenia is induced by agents such as Dicumarol, salicylate, or sulfanyl compounds, the arterial and venous plasmas are equally affected. This is also true of prothrombinopenia arising spontaneously in consequence of debilitating disease. Hence, the normally existing relationship between arterial and venous plasma is unaffected by the development of prothrombinopenia.

Arterial and venous blood may be used interchangeably for estimation of prothrombin time.

The work was conducted in part at the Home of the Daughters of Israel, New York City, through the generous cooperation of Dr. Julius Raab.

The author wishes to thank the following physicians for their assistance in obtaining blood samples: Dr. Robert C. Murphy, Jr. (St. Lukes Hospital); Dr. Sylvan M. Stillman (Bellevue Hospital).

Dicumarol was supplied by Dr. R. K. Richards of Abbott Laboratories, North Chicago, Ill. Miss Frances Kaufman gave technical assistance.

*Moderate prolongation: between 1½ and twice normal. Marked prolongation: greater than twice normal.[14]

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LABORATORY METHODS

GENERAL

USE OF TERTIARY BUTYL ALCOHOL IN BACTERIOLOGIC STAINING PROCEDURES*

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BACTERIOLOGISTS and clinical pathologists may desire satisfactory substitutes for certain reagents which are becoming increasingly expensive and difficult to obtain.

Tertiary butyl alcohol was introduced into microtechnical procedures by Johansen,[1] who subsequently developed methods for its use as a dehydrating agent in plant microtechnique.[2-4] Stowell[5, 6] found this reagent satisfactory for dehydration of a large variety of mammalian tissues and recommended its use in place of ethyl alcohol to help conserve reagents which are vital to our war industries.[7]

Various solvents such as methyl, ethyl, and isopropyl alcohols and acetone have been used as decolorizing agents in making Gram stains of bacterial films. In the present study over five hundred preparations of pure cultures, mixed suspensions, unknown organisms isolated at autopsy, urethral pus, and tuberculous sputum were stained. Except for sputum, the films were stained by the routine Gram staining procedure used in this laboratory and also by a procedure substituting tertiary butyl alcohol for ethyl alcohol as a stain solvent and decolorizing reagent; likewise, in the destaining of nonacid-fast organisms in sputum, tertiary butyl alcohol was substituted for ethyl alcohol.

EXPERIMENTAL

Preparation of Solutions.—The primary Gram stain was prepared by dissolving 2 Gm. of crystal violet (or gentian violet, Coleman-Bell) in 20 c.c. of tertiary butyl alcohol. This was then mixed with 80 c.c. of a 1 per cent aqueous solution of ammonium oxalate and filtered. Gram's iodine solution (0.33 per cent iodine, 0.67 per cent potassium iodide in distilled water with a small lump of calcium carbonate added to stabilize the solution) was used as the mordant. Ethyl alcohol (95 per cent) or tertiary butyl alcohol was used to destain the Gram negative organisms. The counterstain was a 2.5 per cent aqueous solution of safranine.

Carbolfuchsin solution employed in a modified Ziehl-Neelsen technique was prepared by dissolving 0.3 Gm. of basic fuchsin in 10 c.c. of tertiary butyl alco-

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hol and mixing this with 90 c.c. of a 5 per cent aqueous solution of phenol. A 5 per cent solution of nitric acid in tertiary butyl alcohol or in ethyl alcohol (95 per cent) was used for decolorizing.

Procedures and Results.—In order to determine the proper time relations when tertiary butyl alcohol was used as a decolorizing agent, *Staphylococcus aureus* and *Escherichia coli* were selected as test organisms. Several bacterial films made in the usual manner were stained by the modification of Gram's procedure used satisfactorily in this laboratory for routine work (crystal violet, iodine solution, 95 per cent ethyl alcohol, and safranine applied in order, ten to fifteen seconds each, followed by washing in tap water). Similar films were prepared and stained in order to test the action of various concentrations of tertiary butyl alcohol as a destaining agent. Dilutions of tertiary butyl alcohol were prepared, and each was used in several series of stains in which the time of application of the primary stain, the iodine solution, or the decolorizing agent was varied. For example, in one series the primary stain and iodine solution were applied for a constant time and the period of destaining was altered for individual slides. Each slide was compared with one or more stained by the routine method using ethyl alcohol. When a satisfactory time for decolorizing was determined, other series were studied in which the time of primary staining or application of iodine solution was altered. These slides, likewise, were compared with routine stains. The series were repeated with each of the aqueous dilutions of tertiary butyl alcohol. In general, the periods of time (ten to sixty seconds) during which the primary stain or the iodine solution was applied made little difference in the results.

With 10, 20, and 30 per cent solutions of tertiary butyl alcohol the results were variable and unsatisfactory. When applied as long as five minutes, 50 per cent or less of the Gram negative forms were properly decolorized. Films which were decolorized with 40, 50, or 60 per cent tertiary butyl alcohol showed better differentiation between Gram negative and positive organisms, but variable numbers of the Gram negative forms were not destained unless the tertiary butyl alcohol was applied for two minutes or longer. Under these conditions, however, the Gram positive organisms in the thinner portions of the films were slightly decolorized. This was true especially if the iodine solution had been prepared several weeks previously.

After further studies it was found that tertiary butyl alcohol in concentrations of 60, 70, 80, or 90 per cent was satisfactory for destaining. Dependent on certain factors such as thickness of the film, the time of destaining, and the stability of the iodine solution, Gram negative organisms were properly decolorized and differentiation was good. The most satisfactory results were obtained when thin bacterial films were stained in the following manner:

1. Crystal violet solution, ten seconds; wash in tap water.
2. Iodine solution, ten seconds; wash in tap water and shake slide free of water.
3. Eighty per cent tertiary butyl alcohol, sixty to ninety seconds; wash in tap water.
4. Safranine solution, ten to fifteen seconds; wash and dry.

If thin, even films were prepared, 60 per cent and 70 per cent concentrations of tertiary butyl alcohol differentiated Gram negative forms well but less satisfactorily than did the procedure outlined above. Stains prepared according to this method were compared with those made by the routine procedure (using 95 per cent ethyl alcohol as a decolorizing agent) in identifying organisms isolated from nearly one hundred patients at autopsy. During the course of these studies the following organisms were cultured and satisfactorily stained by the method outlined: *Proteus vulgaris, Serratia marcescens, Staphylococcus aureus* and *albus, Escherichia coli, Alkaligenes faecalis,* streptococci, *Actinomyces hominis,* diphtheroids, *Sarcina lutea, Neisseria catarrhalis,* unidentified sporulating rods, *Pseudomonas aeruginosa,* and two type cultures of Salmonella.

In applying this method to several smears of urethral pus from different patients with acute gonorrhea, it was observed that, except with very thin smears, the time of destaining should be extended to two or three minutes. As other workers have indicated, preparations were satisfactory when pus cells were stained well with the counterstain.

A 5 per cent solution of nitric acid in 50 to 80 per cent tertiary butyl alcohol was satisfactory for decolorizing nonacid-fast bacteria in specimens of tuberculous sputum. Excellent differentiation was obtained by the application of steaming carbolfuchsin for five minutes, followed by destaining for one to two minutes before applying the counterstain.

DISCUSSION

The procedure outlined in this study is not proposed to supplant methods now used in preparing Gram stains, but rather to suggest a method which may be employed satisfactorily in the event that reagents now in common use become too expensive or difficult to obtain. Tertiary butyl alcohol, therefore, may be of importance in bacteriologic staining procedures, since it yields satisfactory results and it is a surplus by-product in the oil industry. According to sales agents[8] of the manufacturer, the supply of tertiary butyl alcohol is plentiful, and it may be obtained without any difficulty. This has been confirmed by the War Production Board.[9] In contrast, the supply of normal and secondary butyl alcohols is limited owing to their important uses in war industries. In the United States most of the tertiary butyl alcohol is marketed as one anhydrous grade produced by one oil company. Retail prices, however, show considerable variation, some firms asking about ten times the wholesale cost. On the basis of comparative prices of 55-gallon drum lots, tertiary butyl alcohol, which is tax-free, is only slightly more expensive than the average price of 95 per cent ethyl alcohol without the added tax.

Pure tertiary butyl alcohol, $(CH_3)_3$—C—OH, although said to be three times as toxic as ethyl alcohol, should be safe for use in the laboratory if ordinary precautions are taken to avoid prolonged exposure to high concentrations of the alcoholic vapors. The compound melts at 23 to 25.6° C., and as traces of water are taken up the melting point is lowered. When the melting point is above 23° C., at least 99.4 per cent of the reagent is present. If the room temperature is below the melting point of the alcohol, the stock bottle may be left in a warm place such as the top of an oven or near a radiator. Tertiary butyl

alcohol is miscible with most reagents in common use in the laboratory, including water, ethyl alcohol, ether, xylene, benzene, and chloroform.

SUMMARY

Tertiary butyl alcohol, a readily available, reasonably priced reagent, is satisfactory as a decolorizing agent in modifications of the Gram and Ziehl-Neelsen staining procedures. Should commonly used reagents become too expensive or unobtainable, tertiary butyl alcoohl may be used in these methods.

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A NEW AND RAPID STAINING METHOD FOR GRAM-POSITIVE AND GRAM-NEGATIVE ORGANISMS IN FROZEN AND PARAFFIN SECTIONS

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CHRISTIAN GRAM[1] in 1884 devised his method for the demonstration of bacteria in tissue sections. Weigert's[2] modification of Gram's method has been useful for the demonstration of fibrin and bacteria and is still widely used, but both methods demonstrate only gram-positive organisms.

Since then, numerous attempts have been made to demonstrate both gram-positive and gram-negative organisms in paraffin sections of tissues that had been fixed in Zenker's solution or in formaldehyde solution, and methods accomplishing this purpose have been reported by MacCallum,[3] Lyon,[4] Lillie,[5] Brown and Brenn,[6] Rudnikoff and Stawsky,[7] Glynn,[8] and others.

In these modifications, the staining solutions as devised by Gram remained practically unchanged. No attempt has been made to devise a new method by the use of some other dye or chemical, and no recommendations have been made as to the applicability to frozen sections.

The solutions used by Gram and used in the modifications of his method are: gentian violet, methyl violet, and crystal violet, as gram-positive stains; Gram's solution and compound solution of iodine, U.S.P. (Lugol's solution), as

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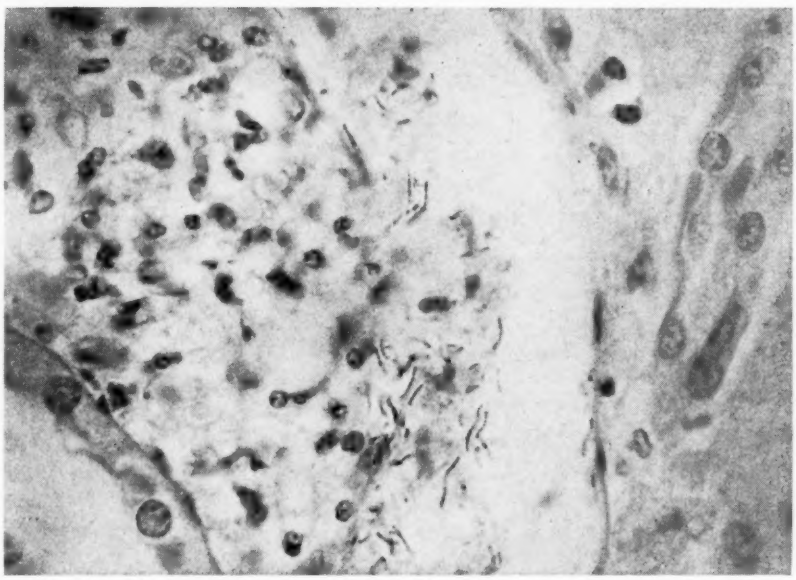

Fig. 1.—Streptococcus in kidney, stained blue; ×1620.

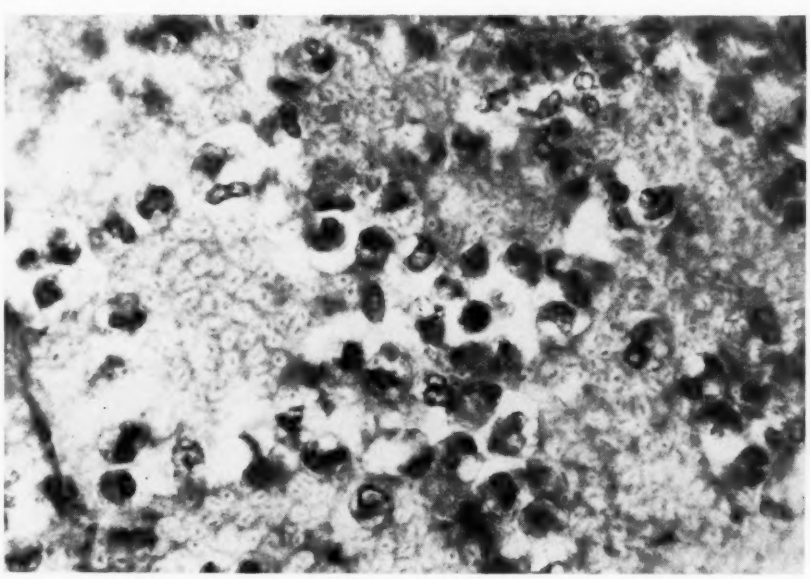

Fig. 2.—Friedländer's bacilli in pneumonia, stained red; ×1620.

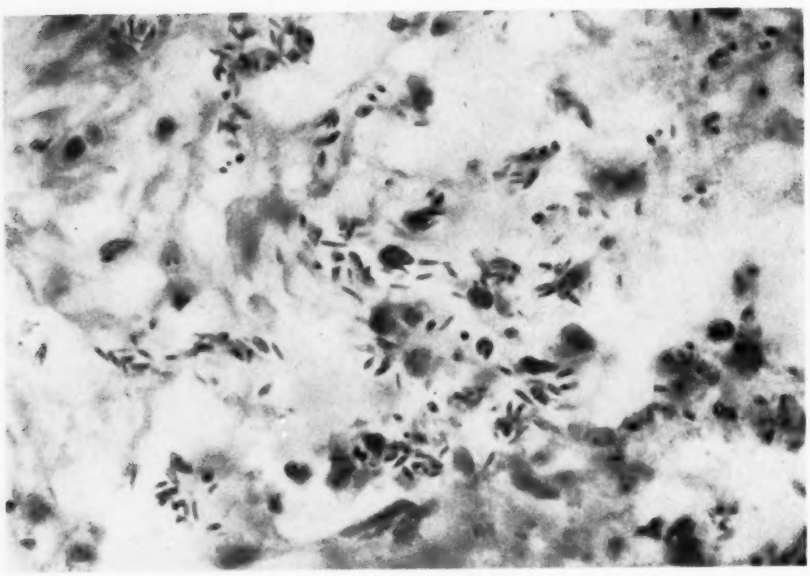

Fig. 3.—Sporotrichum in testicle, stained blue; ×1620.

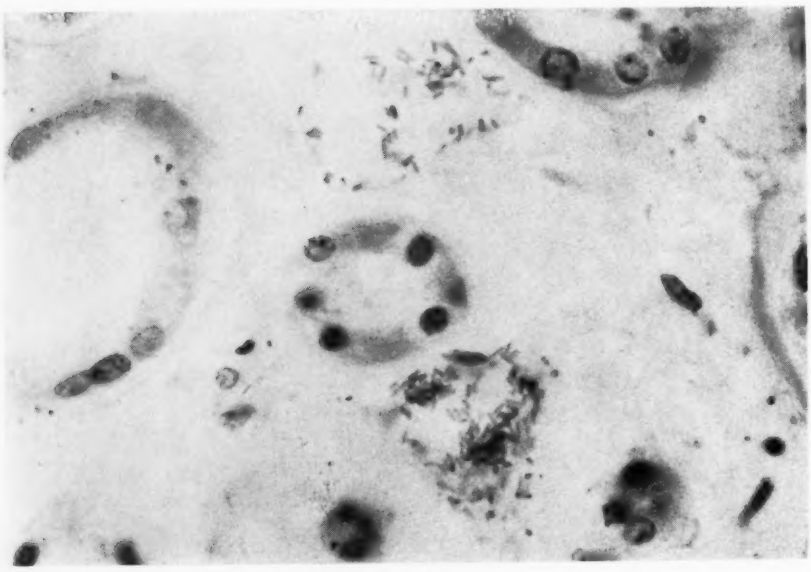

Fig. 4.—Colon bacilli in kidney tubules, stained red; ×1620.

differentiators; rosaniline hydrochloride, basic fuchsin, dilute carbolfuchsin, and safranine as gram-negative stains; and acetone, alcohol, ether, and aniline oil-xylene as decolorizers.

The need for rapid diagnosis led me to investigate the usefulness of a different dye and different solutions. A new and simple method was devised which is easily applied to frozen and paraffin sections of formaldehyde fixed tissue and which gives satisfactory simultaneous differential staining of gram-positive and gram-negative organisms. For example, four different sections (frozen or paraffin) mounted on the same slide, one of each of a kidney containing monilia, a lung with Friedländer's bacilli, a testicle containing sporotrichum, and a kidney with colon bacilli, were stained, with successful differentiation of the organisms by the method described. The technique has also been used on tissues containing streptococci, Welch bacilli, influenza bacilli, actinomyces, meningococci, gonococci, diphtheria bacilli, and Negri bodies. Similar results are obtained in another method recently described by me,[9] but the time and material required has been greatly decreased in the present technique.

In this new method, the solutions used are: methylene blue as a gram-positive stain, fuchsin-creosote as a gram-negative stain, creosote-xylene as a differentiator.

THE FROZEN SECTION METHOD

1. Prepare mounted frozen sections at 7 to 10 microns in the usual manner.
2. Stain for three minutes with alkaline methylene blue (Loeffler's).
3. Wash in tap water and dehydrate rapidly with three applications of anhydrous isopropanol or absolute ethyl alcohol.
4. Differentiate rapidly with creosote-xylene (1 part creosote plus 2 parts xylene), agitating slide constantly for five to ten seconds.
5. Pour off and apply creosote-fuchsin (2.5 c.c. of a 6 per cent alcoholic basic fuchsin added to 50 c.c. of creosote-xylene), agitating slide about 15 to 20 times, changing the solution once.
6. Blot and apply creosote-xylene 2 or 3 times, agitating the slide constantly for even decolorization or until most of the excess red color leaves the section.
7. Blot, clear for two minutes in xylene and mount in gum dammar.

FOR PARAFFIN SECTIONS

Deparaffinize section with two applications of xylene and two applications of absolute alcohol or isopropanol; bring down to tap water and then proceed with the method described.

With the use of this method, nuclei are red, gram-positive organisms blue, gram-negative organisms red, devitalized gram-positive organisms red, monilias and actinomyces blue, Negri bodies bright red with bluish chromatin bodies, and fibrin sometimes blue and sometimes red.

All the staining solutions are stable.

SUMMARY

A new, simple, and rapid method for the demonstration of gram-positive and gram-negative organisms in frozen and paraffin sections of formaldehyde fixed tissues is presented. It has the particular advantage of demonstrating the

devitalized forms of gram-positive organisms. The staining solutions used are: methylene blue, fuchsin-creosote, creosote-xylene.

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LITMUS PAPER DISPENSER*

E. E. Beohm, A.B., Philippi, W. Va.

IN USING litmus paper to test the acidity of urine the usual method of taking a strip from a vial and tearing off a piece tends to be wasteful of paper and is otherwise unsatisfactory.

Fig. 1.

The use of vials filled with cut squares to be removed by the fingers likewise has its disadvantages in that the fingers must be dry; else some of the paper may be ruined, etc.

*From The Myers Clinic Laboratory, Philippi, W. Va.
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To eliminate these difficulties we have devised a litmus paper dispenser, which has proved to be a distinct aid in our laboratory. A Petri dish is used to hold squares of litmus paper (best cut from one strip at a time so that they will not tend to stick together). The dish is divided into two compartments for red and blue paper by a piece of 7 mm. glass rod, cemented to the glass by household cement. A pair of tweezers is used to pick up the squares of paper.

Fig. 1 is a photograph of the dispenser.

THE COMBINING POWER OF STAPHYLOCOCCUS TOXOID AS AN IDENTITY TEST*

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MANY favorable reports on the use of staphylococcus toxoid in the treatment of localized staphylococcal infections have now appeared (Dolman,[1] Connor and McKie,[2] Whitby,[3] and Murray[4]). The control of innocuity and antigenic potency of this product is governed adequately by the regulations of the National Institute of Health relating to staphylococcus toxoid. The same regulations also require identity tests, where possible, for all biologicals.

It is the purpose of this communication to outline a procedure which has proved satisfactory over a period of several years as an identity test for staphylococcus toxoid.

The method employed for identifying staphylococcus toxoid consists essentially of its ability to combine with staphylococcus antitoxin in the presence of rabbit red blood corpuscles as an indicator. It is necessary to have staphylococcus alpha toxin of suitable hemolytic power for rabbit red cells.

PROCEDURE

The first step in the procedure is to determine the lethal dose of the toxin, and this is done in the following manner. Serial dilutions of toxin are made in ten serologic tubes so that Tube 1 contains 1 c.c. of a 1:10 dilution and Tube 10 contains 1 c.c. of a 1:100 dilution. Standard staphylococcus alpha antitoxin is added to the tubes in constant quantity so that each c.c. contains 1 International Unit. One c.c. of 4 per cent washed rabbit erythrocytes is added to each tube and the final volume is made up to 4 c.c. with physiologic saline. The tubes are incubated in a water bath for sixty minutes at 37° C. A sample titration is found in Table I.

The 50 per cent end point is observed in Tube 7, which contains staphylococcus toxin in a dilution of 1:25, and the lethal dose is considered as 0.04 c.c.

In order to establish the identity of the unknown material (staphylococcus toxoid?), the titration is altered somewhat so that the material under test is con-

*From the Research and Biological Laboratories, Ayerst, McKenna and Harrison, Ltd., Montreal.

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tained in the hemolytic system. One c.c. of the material may be diluted 1:2 or 1:4 and is added in constant quantity. A sample titration is found on Table II.

Thus it is observed that by the addition of the unknown material to the hemolytic system, the 50 per cent end point was moved from Tube 7 to Tube 9, proving that combination has taken place between the unknown material and staphylococcus antitoxin. The only variable between the two hemolytic systems being the unknown in the second titration; therefore, the conclusion can justifiably be drawn that the unknown material must be staphylococcus toxoid.

TABLE I

TITRATION TO DETERMINE LETHAL DOSE OF STAPHYLOCOCCUS ALPHA TOXIN

TUBE NO.	1	2	3	4	5	6	7	8	9	10
	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.
Staphylococcus Toxin 1:10 Dil.	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
Saline	1.0	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9
Staphylococcus Antitoxin 1 I.U.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Rabbit red blood cells 4 per cent	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Results per cent hemolysis	100	100	100	100	100	90	50	25	0	0

TABLE II

TITRATION TO DETERMINE COMBINING POWER OF MATERIAL (STAPHYLOCOCCUS TOXOID) UNDER TEST

TUBE NO.	1	2	3	4	5	6	7	8	9	10
	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.
Unknown 1:4 Dil.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Staphylococcus Toxin 1:10 Dil.	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
Saline	–	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
Staphylococcus Antitoxin 1 I.U.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Rabbit red blood cells 4 per cent	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Results per cent Hemolysis	100	100	100	100	100	100	100	90	50	0

CONCLUSIONS

The procedure described for the identification of staphylococcus toxoid is simple and efficient. Since the lethal dose of the staphylococcus toxin must be determined as one of the mandatory tests in the establishment of the potency of the material, no added substances are necessary in the performance of the identity test save that of the material under test.

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RAPID BIOLOGICAL METHOD FOR THE APPROXIMATE DETERMINATION OF ACETYLCHOLINE AND POTASSIUM IN BODY FLUIDS*

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THE acetylcholine content of body fluids is usually determined by biological methods. Since potassium affects the result and its content in the body fluids varies greatly, it is necessary to differentiate its effects from those of acetylcholine. The differentiation is possible by the combined application of two or more of the methods in common use. Some of the more common methods are:

(1) Chemical determination of the acetylcholine content.
(2) Chemical determination of the potassium content.
(3) Comparison of the effect of various dilutions of the body fluid on a sensitive tissue.
(4) Precipitation of potassium by permutit (Lánczos.[1]).
(5) Comparison of the effect of the unknown body fluid on the biological test objects before and after eserinization.
(6) Comparison of the effect of the unknown body fluid on the biological test objects before and after atropinization.

Such a combination, while giving reliable results, requires much time.

This paper describes a rapid method for the approximate determination of acetylcholine and potassium. The effects of the procedure are fully reversible, making a check possible. The method is based on the property of calcium to counteract the effect of potassium. The rectus abdominis muscle of the frog was used as the test object, as suggested by Riesser,[2] and Chang and Gaddum.[3]

METHODS

The rectus abdominis of the frog was excised and suspended in a muscle chamber containing 20 c.c. of Ringer's solution (114 mM NaCl, 2 mM KCl, and 1.8 mM $CaCl_2$). After one hour the Ringer's solution was replaced for thirty seconds by a test solution containing substances causing contraction of the muscle. The height of contraction was registered by an isotonic lever on a kymograph. The muscle was then washed for ten minutes in Ringer's solution and the procedure repeated until three successive exposures to a test solution gave similar responses. At this point the counterbalance on the lever was adjusted so that the height of the reading on the kymograph was the same in the various experiments. Then the muscle was exposed to a series of test solutions for thirty seconds and washed with Ringer's solution for ten minutes.

In order to eserinize the muscle it was immersed for thirty minutes in a 0.0004 per cent physostigmine salicylate solution. Between exposures to the

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various test solutions the eserinized muscle was washed first with Ringer's solution for five minutes and then with physostigmine for five minutes.

The test solutions used were:

(1) Acetylcholine solution containing 0.1 to 0.01 mg. per 100 c.c. with uneserinized muscles and 0.01 to 0.001 mg. per 100 c.c. with eserinized muscles.
(2) 20 to 60 mM potassium.
(3) Both acetylcholine and potassium.

Calcium was present in the various solutions in concentrations of from 0 to 180 mM. The solutions were rendered isotonic with Ringer's solution by the addition of NaCl. The pH was corrected to 7 when necessary by the addition of acid or basic phosphate. Air was bubbled through the solutions in the muscle chamber during the experiments.

The results were tabulated in millimeters of excursion of the lever as measured from the records.

TABLE I

ACETYLCHOLINE AND POTASSIUM CONTRACTIONS OF THE RECTUS ABDOMINIS MUSCLE IN RELATION TO THE CALCIUM CONCENTRATION

SUBSTANCE	NO. OF EXPTS.	AVERAGE CONTRACTION IN PER CENT OF THAT OBTAINED IN RINGER'S SOLUTION (Ca 1.8 mM) FOLLOWED BY STANDARD ERROR OF MEAN				
		18 mM $CaCl_2$	36 mM $CaCl_2$	54 mM $CaCl_2$	90 mM $CaCl_2$	180 mM $CaCl_2$
Acetylcholine*	25	90 ± 1.0	85 ± 0.9		80 ± 1.1	
20 mM Potassium	25	35 ± 0.9	16 ± 0.5		11 ± 1.1	
40 mM Potassium	25	55 ± 0.9	35 ± 0.9		11 ± 1.0	4 ± 0.7
60 mM Potassium	10			36 ± 1.1	18 ± 1.0	5 ± 1.0
Acetylcholine and 20 mM Potassium	10	65 ± 1.0			56 ± 1.0	
Acetylcholine and 40 mM Potassium	10		65 ± 1.0		55 ± 1.0	

*For concentration of acetylcholine see text.

RESULTS

The Effect of Acetylcholine in the Presence of Various Concentrations of Calcium.—The results, summarized in Table I and Fig. 1, show that an increase of the calcium content of the acetylcholine solution from 1.8 to 18 mM decreased the height of contraction caused by acetylcholine by about 10 per cent; and an increase of the calcium content to 90 mM decreased the height of contraction by about 20 per cent. Thus, varying the concentrations of calcium in the test solution had but little effect on the height of contraction caused by acetylcholine. This was true with both eserinized and uneserinized muscle, the only difference being in the absolute concentration of acetylcholine necessary to produce the contraction.

The Effect of Potassium in the Presence of Various Concentrations of Calcium.—The results are given in Table I and Fig. 1. They are expressed as a percentage of the response to solutions containing respectively 20, 40, and 60 mM potassium in the otherwise standard Ringer's solution (Ca, 1.8 mM). The

effectiveness of all these potassium solutions fell off rapidly as the calcium concentration was increased. At the higher potassium concentrations slightly more calcium was required to produce a given reduction in the response, but for all three concentrations 180 mM calcium caused a reduction in the average response to 5 per cent or less of the control value.

The Effect of Acetylcholine Plus Potassium in the Presence of Various Concentrations of Calcium.—The contracting effect of a solution containing acetylcholine and potassium is greater than one containing acetylcholine or potassium alone.

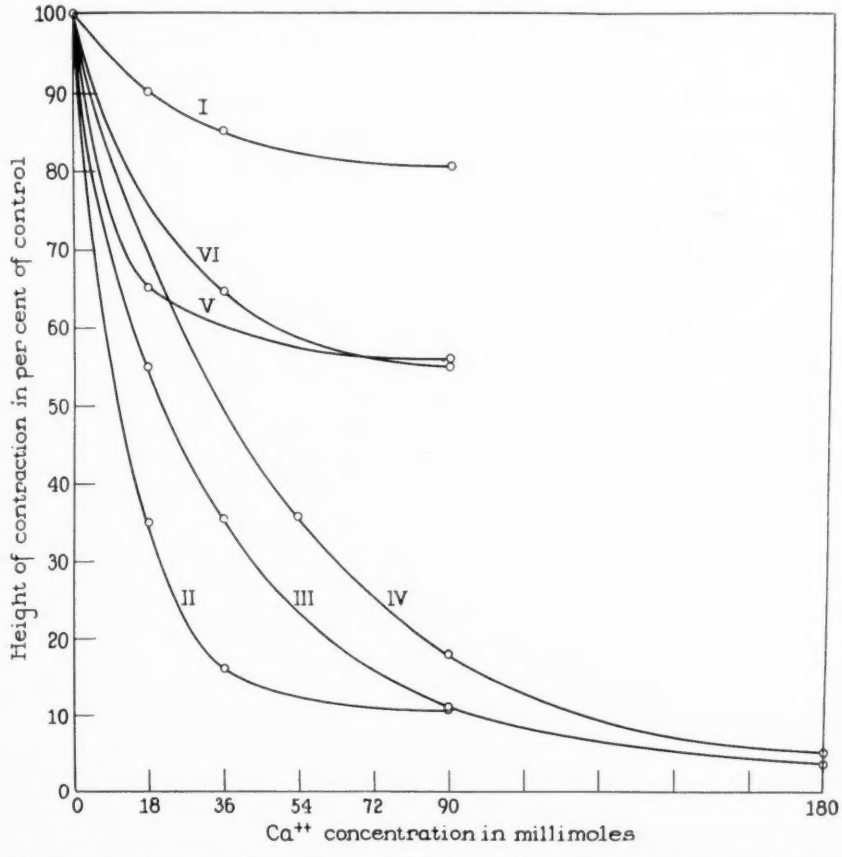

Fig. 1.—Influence of calcium on the contraction of the rectus abdominis muscle caused by acetylcholine and potassium. Values are expressed in percentage of the control response with muscle in Ringer's solution (1.8 mM Ca). The height of contraction in per cent is plotted against the calcium concentration in millimols. I, Acetylcholine (for concentration see text). *II*, 20 mM Potassium. *III*, 40 mM Potassium. *IV*, 60 mM Potassium. *V*, Acetylcholine and 20 mM Potassium. *VI*, Acetylcholine and 40 mM Potassium.

In the first series of experiments the muscle response to a solution containing 0.1 to 0.01 mg. acetylcholine per 100 c.c., 20 mM potassium, and 1.8 mM calcium was recorded. An increase in the calcium content to 18 mM decreased the height of contraction by about 35 per cent, and an increase of the calcium concentration to 90 mM caused a 44 per cent decrease. In the second series of experiments the muscle was exposed to a solution containing acetylcholine (0.1 to 0.01 mg. per 100 c.c.), 40 mM potassium and 1.8 mM calcium. An increase of the calcium con-

centration to 36 mM decreased the height of contraction by 35 per cent, and an increase of the calcium concentration to 90 mM caused a 44 per cent decrease. This effect was observed with both eserinized and uneserinized muscle, the only difference being the concentration of acetylcholine required to produce the contraction.

A series of experiments paralleling the experiments described above in which proteins were added to the test solution have been carried out with results similar to those summarized in Table I and Fig. 1.

The technique described may be used for an approximate determination of the acetylcholine and potassium content of an unknown solution by the following procedure: First, the eserinized rectus abdominis of the frog is immersed in the unknown solution, the height of contraction registered. Then to an aliquot, 90 mM calcium is added and the contracting effect of this solution is measured. If the increase in the concentration of calcium does not modify significantly the height of contraction, it may be assumed that the original solution contained acetylcholine and little or no potassium. If on the other hand the height of contraction is decreased by 80 or 90 per cent, the solution may be assumed to contain potassium but no acetylcholine. If an intermediate result is obtained, it may be assumed that both potassium and acetylcholine are present in the unknown solution.

The method can be used for quantitative estimation of the acetylcholine or potassium content of an unknown solution:

If the solution contains acetylcholine alone, the acetylcholine content can be estimated by comparing the height of contraction of the muscle immersed in the unknown solution with the height of contractions caused by known solutions.

If the solution contains potassium without acetylcholine, a determination of the amount of potassium is possible by varying the calcium concentration of the solution. If the addition of 36 mM calcium decreased the height of contraction by about 80 per cent, it may be assumed that the unknown solution contains about 20 mM potassium. If the decrease is 65 per cent, the original solution probably contains 40 mM potassium, and similarly if the decrease is 50 per cent, the solution probably contains 60 mM potassium.

With a solution that contains both acetylcholine and potassium a similar procedure can be carried out. For example, if the addition of 18 mM calcium to the unknown solution results in a 35 per cent decrease in the height of contraction, it may be assumed that the solution contained about 20 mM potassium. Similarly if the addition of 36 mM calcium to the unknown solution results in a 35 per cent decrease of the height of contraction, the solution contained about 40 mM potassium. After estimation of the concentration of potassium of the unknown fluid, the acetylcholine content can be estimated by the usual method indicated above. The height of contraction caused by the unknown solution must be compared with the height of contraction produced by known solutions of acetylcholine dissolved in the same concentration of potassium as the unknown solution.

The potassium content of the unknown solution may also be determined by plotting the height of contraction of the rectus abdominis, as expressed in percentage of the first contraction, against the increase of the calcium content of

the fluid. By a gradual increase of the calcium content from zero to a high concentration, the curve obtained is a hyperbola. As the ratio of the K/Ca content of the solution decreases towards unity, the curve falls sharply. As the ratio becomes less than unity, the curve starts to level out and approximate asymptotically the base line.

The body fluids contain buffers, most of which form insoluble salts with calcium; therefore, a correction must be made for the amount of calcium precipitated. This can be accomplished by adding calcium in small amounts until no further precipitate is formed. Any further amounts of calcium added remain for the most part in solution and are available to counteract the effect of potassium. The pH of all solutions must be corrected to the same level, since the height of contraction varies with the pH.

Immersion of the muscle for a few minutes in Ringer's solution is sufficient to reverse the effect of a few minutes' immersion in calcium.

The method was tested for urine. Acetylcholine (0.1 to 0.001 mg. per 100 c.c.), potassium (20 to 50 mM) or both acetylcholine and potassium were dissolved in urine and the added amount determined. The difference between the amount of substances added and that estimated by the muscle test was less than ±15 per cent.

DISCUSSION

This paper offers a method of determining the approximate content of acetylcholine and/or potassium in an unknown solution based on the effect of calcium in counteracting the effect of potassium on the rectus abdominis muscle.

The method is only approximate because of the following sources of error:

(1) The calcium-potassium antagonism on muscle contraction is not absolute.
(2) The original calcium ion content of the unknown solution is not known.
(3) It is difficult to estimate the exact amount of ionized calcium in case some of the added calcium precipitates.
(4) The unknown fluid might contain other substances which cause muscle contraction and which are more or less influenced by calcium.

The above studies prove that the effect of acetylcholine as a contraction-producing agent is somewhat modified by an increase in the concentration of calcium in the surrounding fluid. They also prove that the contraction of the muscle is due not to an excess of potassium per se, but to an increase of the K/Ca ratio. The calcium-potassium antagonism is not a simple relationship, since exceedingly large amounts of calcium are necessary to counteract completely the effect of potassium.

SUMMARY

(1) A biologic method for the rapid estimation of acetylcholine and/or potassium content of body fluids is offered. The method is based on the antagonism between calcium and potassium on the contraction of the rectus abdominis muscle of the frog.

(2) The contracting effect of acetylcholine on the muscle is but slightly influenced by a large excess of calcium.

(3) The contraction caused by potassium can be prevented almost completely by a large amount of calcium.

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A STUDY OF PRESERVED COMPLEMENT*

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THE most uncertain factor in complement fixation tests has always been the complement itself, since it is practically impossible to predict how long any particular lot of freshly drawn guinea pig serum will retain its potency. Especially distressing is the instability of complement diluted for tests; and it is perhaps because of this instability that some attempts at complement fixation have met with little success.

During recent years, however, several workers have attempted to reduce the factor of uncertainty by various methods of preservation. Ginsburg and Kalinin[1] reported satisfactory preservation of guinea pig complement for three to four months by addition of 100 mg. sodium chloride and 40 mg. boric acid per c.c. of serum. The method used in the present study is based on the one described by these authors and differs mainly in that 80 mg. instead of 100 mg. of sodium chloride were used.

PREPARATION OF PRESERVED COMPLEMENT

For each pool of complement serum, 8 to 10 c.c. of blood were drawn by cardiac puncture from each of four or more healthy adult guinea pigs, under ether anesthesia. The blood was collected separately in sterile Petri plates, left at room temperature for an hour, and in the cold room four to twenty hours. (Petri plates were used to allow greater surface for evaporation of the ether with which the blood becomes saturated during anesthesia.) The clots were carefully removed, avoiding hemolysis. The clear sera were obtained by the minimum amount of centrifugation and placed immediately in the cold room. Within twenty-four hours after bleeding the animals, a portion of each serum was removed for titration according to the technique given later. In this titration, unless there was complete hemolysis of one unit of sensitized sheep cells in the presence of less than 0.05 c.c. of the 1:15 diluted complement, the latter was not included in the pool.

The satisfactory sera were pooled in a large sterile test tube fitted with a cotton plug. After the volume had been determined, 80 mg. of sodium chloride

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and an excess (40 mg.) of pulverized boric acid were added per c.c. of serum. If it were not possible to titrate the sera within twenty-four hours, proportionate amounts of the salts were added to the individual sera; the mixtures were shaken till it was certain that all the sodium chloride had dissolved; and the titrations were done later. Since boric acid dissolves very slowly, the mixture was agitated about three times daily for two days to insure maximum solubility. When the odor of ether was no longer detectable in the serum, the cotton plug was replaced by a cork stopper to prevent further evaporation.

PREPARATION OF THE REAGENTS FOR COMPLEMENT TITRATIONS

Washed Sheep Blood Cells.—The cells of freshly drawn sheep blood were washed four times in large volumes of physiologic salt solution, and a 5 per cent suspension was made in saline.

Sensitized Sheep Cells.—The sheep cells were sensitized by mixing the required volume quickly and thoroughly with an equal volume of amboceptor diluted according to titer. The suspension was again agitated every five minutes for fifteen minutes, at which time it was considered ready for use and was discarded after one and one-half hours. Of this suspension 0.2 c.c. were used as one unit.

Titration of Amboceptor.—The amboceptor titer was determined as the highest dilution of amboceptor to the nearest 100, producing complete hemolysis of the cells in the presence of 0.05 c.c. of 1:15 diluted complement.

Because any slight error in the preparation of dilutions might result in amboceptor of varying potency and because the blood cells of different sheep vary somewhat in fragility, care was taken in so far as possible to standardize these two reagents, thereby leaving the complement as the only significant variable throughout the period of the experiments.

Complement.—Throughout the entire period of this work the complement sera were used at a dilution of 1:15. Dilutions of unpreserved complement were made with physiologic saline. Preserved complement was first made isotonic to normal serum by diluting it 1:10 with distilled water, and then made up to 1:15 with physiologic saline.

TECHNIQUE OF COMPLEMENT TITRATIONS

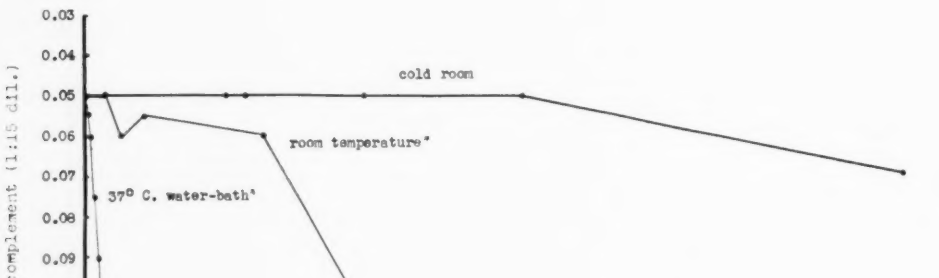
A series of several tubes was set up for each specimen of complement, and various amounts of the diluted complement were pipetted to the bottom of the tubes. To each tube was added 1 unit of sensitized sheep cell suspension. As each rack of tests was completed, it was shaken thoroughly, and placed immediately in a water bath at 37° C. for fifteen minutes. The titer recorded was the smallest volume of diluted complement, to the nearest 0.005 c.c., necessary for complete hemolysis of the sensitized sheep cells, as indicated by simple inspection.

EFFECT OF AGE AND TEMPERATURE ON THE STABILITY OF PRESERVED COMPLEMENT

At varying intervals over a period of sixteen months, complement titrations were made on 29 pooled guinea pig sera of varying ages, accumulated during this time and stored continuously in the cold room at 5° to 8° C.

One of these sera, already two and one-half months in storage, was selected to determine the effect of intermittent exposure to higher temperatures. This complement was divided into three portions, the first of which was kept continuously in the cold room. The second and third portions were also stored in the cold room when not in use; but at some arbitrarily set time before titration, the second portion was transferred to room temperature (25° to 32° C.), and the third to a 37° C. water bath. Then samples of each portion were diluted 1:15 for titrations.

Results.—In considering analysis of Graph I, it must be remembered that the complement used was already two and one-half months old at the first point plotted. The "cold room" curve, which followed a course similar to those obtained for all the pooled sera tested, continued a straight line for twenty-two days more. No test was made between the twenty-second and forty-first days, but tests with other sera indicated that the curve would have continued a straight line to the thirtieth or thirty-fifth day. Hence, preserved complement kept in the cold room would retain its activity practically constant for three to four months.



Graph I.—Effect of temperature on stability of undiluted preserved complement indicated by c.c. of 1:15 dilution required for complete hemolysis of 1 unit of sensitized cells.

The complement was already 2.5 months old at the beginning of the experiment.

*Sera were not left continuously at the temperature specified, but stored in the cold room between test periods.

On the other hand, intermittent exposure of the complement to higher temperatures increased the rate of deterioration, as indicated by the more rapid drop in the other two curves.

EFFECT OF AGE AND TEMPERATURE ON STABILITY OF DILUTED PRESERVED COMPLEMENT

Samples of several preserved sera were diluted 1:15. The dilutions were stored in the cold room and titrated at intervals of approximately twenty-four hours for several days.

One of these diluted sera, selected to determine the effect of intermittent exposure to higher temperatures, was divided into three portions. The first of these was kept continuously in the cold room; the second and third were also

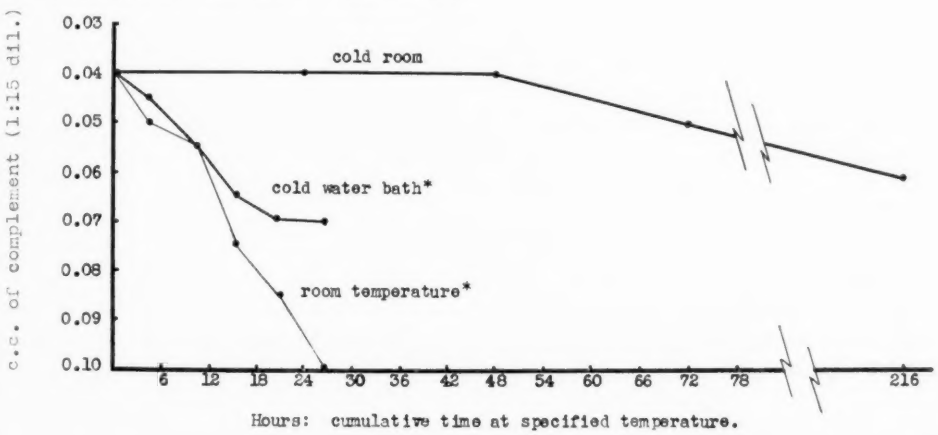
stored in the cold room when not in use. At some arbitrarily set time before titration, the second portion was transferred to a cold water bath (10° to 20° C.) and the third to room temperature (25° to 32° C.). Then samples of each were taken for titration.

Results.—The "cold room" curve in Graph II indicates that the dilution of preserved complement kept continuously in the cold room retained practically constant potency for twenty-four to forty-eight hours. This was also true of the several other diluted sera tested, some of which retained their potency still longer.

On the other hand, intermittent exposure of diluted complement to higher temperatures increased the rate of deterioration as indicated by the more rapid drop in the other two curves.

DISCUSSION

It has long been known that freshly drawn guinea pig complement can no longer be used three or four days after drawing. Furthermore, it has been the experience in the present study that a dilution of unpreserved complement demonstrated appreciable deterioration within eighteen to twenty hours even if kept at cold room temperature.



Graph II.—Effect of temperature on stability of diluted (1:15) preserved complement, indicated by number of c.c. required for complete hemolysis of 1 unit of sensitized cells.

*Sera were not left continuously at the temperature specified, but stored in the cold room between test periods.

From the data presented it seems evident that complement prepared according to the method described will remain satisfactory for a much longer period of time (three to four months) comparing favorably with the complement of Ginsburg and Kalinin. For best results, however, it is necessary to avoid exposure of the complement to temperatures above 5° to 8° C. for any considerable period of time, especially during the first twenty-four hours after obtaining the blood from the guinea pigs; that is, during the time when the salts have not yet been added. The stability of complement seemed to depend in great degree upon its plane of potency at the time the preservatives were added, an occasional pool retaining satisfactory activity for as long as six months. From this it also followed that the stability of the diluted complement depended upon the status of the pool from which it was made. A dilu-

tion of a very poor pool, one that had aged considerably or one that was poor at the very beginning, was likely to be less stable than a dilution of a good pool.

Complement preserved by freezing and drying, according to Flosdorf, Boerner, Lukens, and Ambler,[2] apparently retains its activity for periods as long as three years. The later method of Boerner, Flosdorf, and Lukens,[3] of freezing and drying after addition of sodium acetate and boric acid, also has its advantages. But the advantage of a method like the one described in this study is that it does not necessitate having special apparatus which might be too cumbersome as well as too expensive for a small laboratory. Furthermore, it is rather important to know how the frozen complement behaves after dilution for use. That is, if a dilution of this complement is only as stable as a dilution of fresh complement, it would seem from the results of the present study that there is an added advantage in the salting method.

SUMMARY

Data are presented concerning the nature of complement preserved with sodium chloride and boric acid.

Complement preserved by the method described retains its activity for three to four months. An occasional pool was found satisfactory for as long as six months.

This complement diluted to titer retained its activity almost undiminished for twenty-four to forty-eight hours when kept at 5° to 8° C.

The importance of temperature is stressed, and a cold water bath is suggested for complement (diluted or undiluted) that must be kept out of the cold room for any length of time.

CONCLUSION

The use of sodium chloride and boric acid in the manner described is a satisfactory method of preserving guinea pig complement. The use of complement so preserved should tend to increase the uniformity and dependability of the results of complement fixation tests.

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AN IMPROVED FEEDER FOR GUINEA PIGS*

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BETHESDA, MD.

THE frequent necessity for accurately measuring the quantity of pellet food consumed by guinea pigs on experiments led to the development of a type of feeder which has been in use in this laboratory for nearly a year. This device offers several advantages over the old style of open box. (1) Contamination of food is reduced to a minimum since the feed opening is adjustable to the size of the head. (2) Very little food is lost since the animal has to insert its head

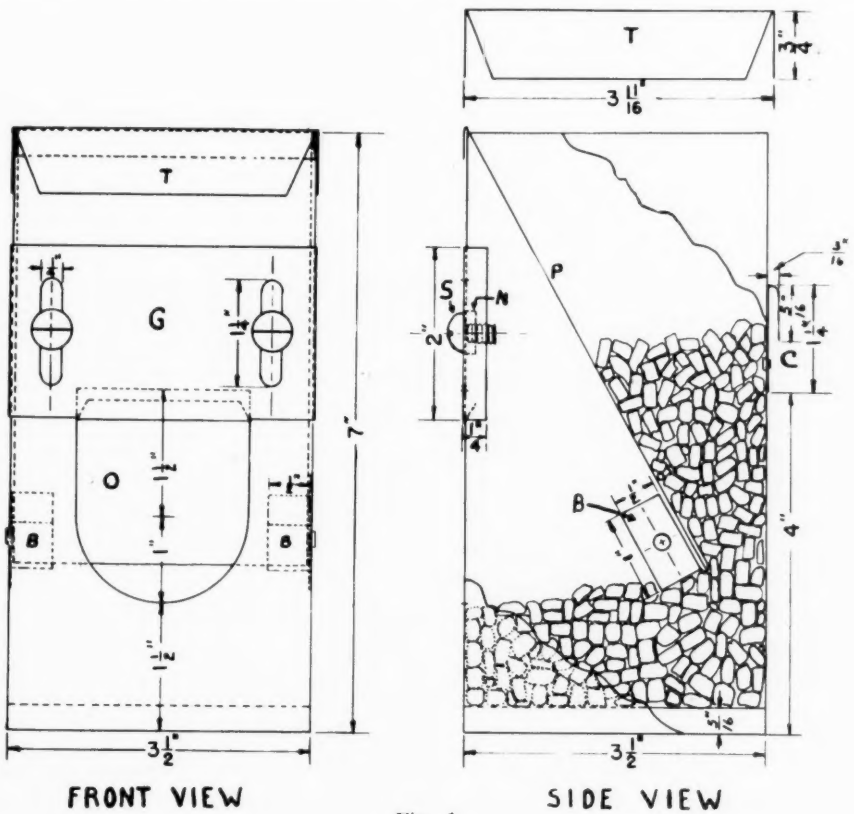

Fig. 1.

into the opening in order to secure food. (3) The capacity of the feeder, about 550 Gm. of pellet food, is sufficient for four or five animals for several days. (4) By reducing contamination and food loss the use of these feeders results in a considerable decrease of food cost. This saving should compensate for the difference in cost between these feeders and open cups.

*From the Division of Industrial Hygiene, National Institute of Health, United States Public Health Service, Federal Security Agency.
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Although feeders made of wood or masonite are serviceable for a limited time, ones constructed of sheet iron are resistant to chewing by the animals. The accompanying drawing shows details and dimensions for feeders made of No. 22 gauge galvanized iron. The box, consisting of four sides with a bottom soldered in, has a smoothly formed feed opening, *O*, the size of which is regulated by the slotted gate, *G*, which is adjustable and can be locked in position by the screws, *S*, and nuts, *N*, soldered to the side. The bottom of the gate is bent upward and backward in order to eliminate any sharp edge which might cut the animal. The plate, P, 5¾ inches long by 3⅜ inches wide, keeps the feed in the hopper and is removable for cleaning. The top of this plate is bent as shown, and the lower part is held in position by the brackets, *B*, soldered and riveted to the sides. The position of these brackets governs the throat size at the bottom of the plate and consequently the flow of pellets. About ¾ inch is satisfactory for this throat width using cylindrical pellets having diameters of about 3/16 inch and lengths ranging from ⅛ to ¼ inch. The feeder is covered with a top, *T*, the edges of which are bent inward so that friction prevents the animal from knocking it off in case it should climb on top. Finally, the clip, *C*, riveted to the back, serves to hold the feeder securely to the cage and prevents upsetting.

We wish to thank Mr. R. R. Groomes, Foreman of the Sheet Metal Shop, for suggestions in the design of this feeding device.

A RAPID METHOD FOR DETERMINING THE SULFONAMIDE DRUG OF CHOICE*

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DURHAM, N. C.

IN SULFONAMIDE therapy, the choice of the drug, its dosage, its effective blood level, its reactions, etc., depend both on the patient and the infecting organism. Bactericidal tests of the patient's organism with the various sulfa drugs are desirable so that the most effective one can be selected as quickly as possible. A preliminary report describing a rapid method for the determination of the drug of choice in cultures of the blood, spinal fluid, nose and throat, sputum, and wounds is presented.

MATERIALS AND METHODS

Sulfanilamide, sulfapyridine, sodium sulfapyridine,† sulfathiazole, sodium sulfathiazole, sulfadiazine, and sodium sulfadiazine were chosen for the experiments because of their adaptability to clinical use. Stock solutions of the drugs were made in beef-infused buffered broth,‡ pH 7.4,[1] using a 100 mg. per cent concentration of sulfanilamide and the sodium salts, and a 15 mg. per 100 c.c. concentration of sulfapyridine, sulfathiazole, and sulfadiazine.

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*From the Department of Bacteriology, Duke University School of Medicine and Duke Hospital.

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†The sodium salts were used so that higher concentration of the drugs could be obtained. Since the same concentration of the drug and that of its sodium salt are not always comparable in their inhibition effects, both forms of the drug were used for these experiments. At the present time, no explanation can be given of this discrepancy, which has been noted in previous experiments.

‡See footnote on page 1621.

Buffered beef-infused (3.5 per cent) agar,* pH 7.4 was used as the stock medium for pouring plates. This was tubed in 8 c.c. amounts, to which were added varying amounts of the broth filtrates of the drugs. The final concentrations of the drugs used in the experiments were 20, 10, and 5 mg. per cent of the sodium salts; 20 and 10 mg. per cent of sulfanilamide, and 5 mg. per cent of sulfapyridine, sulfathiazole, and sulfadiazine.

BLOOD

Fifteen cubic centimeters of the patient's blood are introduced into a flask containing 4 c.c. of 2.5 per cent sodium citrate solution. One cubic centimeter of the citrated blood is added to each tube containing molten agar (cooled to 40° C.), sulfonamide solution, and broth. Control plates contain agar but no sulfonamide. If the patient is receiving chemotherapy, one plate is poured using agar containing 1 mg. per 100 c.c. para-amino-benzoic acid. The cultures are incubated at 37.5° C.

TABLE I

CHART SHOWING INHIBITION OF GROWTH OF PNEUMOCOCCUS, TYPE 27, IN CULTURE OF BLOOD AFTER EIGHTEEN HOURS' EXPOSURE TO SULFONAMIDES

SULFANILAMIDE		SULFAPYRIDINE	SODIUM SULFAPYRIDINE			SULFATHIAZOLE	SODIUM SULFATHIAZOLE			SULFADIAZINE	SODIUM SULFADIAZINE		
10*	20	5	5	10	20	5	5	10	20	5	5	10	20
0	0	++	+++	++++	++++	+++	+++	++++	++++	0	0	0	0

*Milligrams per cent.
++++ = Complete inhibition.
+++ = 75 per cent inhibition.
++ = 50 per cent inhibition.
+ = 25 per cent inhibition.

TABLE II

CHART SHOWING INHIBITION OF GROWTH OF BETA STREPTOCOCCUS IN CULTURE OF NOSE AND THROAT AFTER EIGHTEEN HOURS' EXPOSURE TO SULFONAMIDES

SULFANILAMIDE		SULFAPYRIDINE	SODIUM SULFAPYRIDINE			SULFATHIAZOLE	SODIUM SULFATHIAZOLE			SULFADIAZINE	SODIUM SULFADIAZINE		
10	20	5	5	10	20	5	5	10	20	5	5	10	20
++++	++++	++	++	+++	+++	0	0	0	0	0	0	0	0

After eighteen hours' incubation, if colonies are visible in the control plates, colony counts are made of the controls and the plates containing the drugs. Smears of the colonies are made and stained by Gram's method and the organism is identified. By this method, the drug of choice can be determined at the time the cultures are positive. (See Table I.)

NOSE AND THROAT

The swabs are placed immediately into tubes containing 10 c.c. of beef-infused broth. As much material as possible is washed from the swabs by rolling them against the inside wall of the test tube. One tenth cubic centimeter of the broth is transferred to another tube containing 10 c.c. of broth and mixed thoroughly (Tube 2). The sulfonamide, 1.5 c.c. sterile sheep's blood,

*Broth and agar containing 1 per cent bacto-peptone (Difco brand) were used instead of peptone-free media, as Gay and his co-workers[2] have shown that sulfanilamide is bacteriostatic in the presence of bacto-peptone. When media containing less than 1 per cent bacto-peptone is used, forty-eight to seventy-two hours' incubation of the cultures is required before colonies appear in the plates.

0.1 c.c. of the broth mixture from Tube 2, and sufficient stock broth to bring the volume up to 15 c.c. are added to each tube of molten agar (cooled to 40° C.), and after thorough mixing, plates are poured. Control plates are poured using agar without drug.

After eighteen hours' incubation, the plates are examined for organisms. If present, colony counts are made and the organisms are identified. (See Table II.)

SPINAL FLUID

The spinal fluid is centrifuged at high speed for fifteen minutes. After centrifugalization, the supernatant fluid is discarded, and a smear is made of the sediment and stained by Gram's method. A tube containing 10 c.c. of blood broth is inoculated with two to four loopfuls of the sediment, depending upon the number of organisms present in the smear, and is incubated at 37.5° C. for thirty minutes. The sulfonamide solution, 1.5 c.c. of sterile sheep's blood, 0.1 c.c. of the broth culture of the spinal fluid, and enough stock broth to bring the volume up to 15 c.c. are added to each tube of molten agar (cooled to 40° C.), and after thorough mixing, plates are poured. Control plates are poured using agar without drug. The cultures are incubated at 37.5° C.

TABLE III

CHART SHOWING INHIBITION OF GROWTH OF PNEUMOCOCCUS, TYPE 17, IN CULTURE OF SPINAL FLUID AFTER EIGHTEEN HOURS' EXPOSURE TO SULFONAMIDES

SULFANILAMIDE		SULFAPYRIDINE	SODIUM SULFAPYRIDINE			SULFATHIAZOLE	SODIUM SULFATHIAZOLE			SULFADIAZINE	SODIUM SULFADIAZINE		
10	20	5	5	10	20	5	5	10	20	5	5	10	20
+	+	++++	++++	++++	++++	++++	++++	++++	++++	++	++	++++	++++

TABLE IV

CHART SHOWING INHIBITION OF GROWTH OF STREPTOCOCCUS HEMOLYTICUS IN CULTURE OF SPUTUM AFTER EIGHTEEN HOURS' EXPOSURE TO SULFONAMIDES

SULFANILAMIDE		SULFAPYRIDINE	SODIUM SULFAPYRIDINE			SULFATHIAZOLE	SODIUM SULFATHIAZOLE			SULFADIAZINE	SODIUM SULFADIAZINE		
10	20	5	5	10	20	5	5	10	20	5	5	10	20
++++	++++	++	++++	++++	++++	++++	0	++	++++	++	++	++	++

After eighteen hours' incubation, the plates are examined, the in vitro effectiveness of the sulfonamides is determined, and the organisms are identified. (See Table III.)

SPUTUM

A smear is made of the sputum and stained by Gram's method. One or two loopfuls of the sputum, depending upon the number of organisms present in the smear, are inoculated into a tube containing 10 c.c. of broth. One-tenth cubic centimeter of a 1:100 dilution of the sputum broth mixture is used as the inoculum, and plates are poured, as described above.

After eighteen hours' incubation, the plates are examined for organisms. If colonies are present, the bacteriostatic effect of the sulfonamides is determined and the organisms are identified. (See Table IV.)

WOUNDS

The swabs are placed immediately into a tube containing 10 c.c. of broth. As much material as possible is washed from the swabs by rolling them against

the inside of the test tube. A smear is made of the broth and stained by Gram's method. Dilutions are made according to the number of organisms in the stained smear. One-tenth cubic centimeter of the final dilution of broth is used as the inoculum, and plates are poured, as described above.

After eighteen hours' incubation, the plates are examined, the in vitro effectiveness of the sulfonamides is determined, and the organisms are identified. (See Table V.)

DISCUSSION

The drug of choice can be determined at the time (eighteen to twenty-four hours) the cultures are positive for growth. In that length of time the organisms most likely have not become drug-fast. Various concentrations of the drugs are used, so that the approximate blood level, necessary for therapeutic effectiveness can be estimated at the same time. Identification of the organisms can be made from the control plates of the experiment, so that duplicate cultures are not necessary.

If the patient's blood contained a high concentration of one of the sulfonamides at the time of the experiments, none of the plates may be positive for growth, since the medium does not contain para-amino-benzoic acid, but growth will appear in the plate to which 1 mg. per 100 c.c. para-amino-benzoic acid has been added. This was not encountered in any of our experiments.

TABLE V

CHART SHOWING INHIBITION OF GROWTH OF HEMOLYTIC STAPHYLOCOCCUS AUREUS IN CULTURE OF WOUND AFTER EIGHTEEN HOURS' EXPOSURE TO SULFONAMIDES

SULFANILAMIDE		SULFAPYRIDINE	SODIUM SULFAPYRIDINE			SULFATHIAZOLE	SODIUM SULFATHIAZOLE			SULFADIAZINE	SODIUM SULFADIAZINE		
10	20	5	5	10	20	5	5	10	20	5	5	10	20
0	0	0	0	0	0	++++	0	++++	++++	0	0	0	0

The experiments included cultures of blood, nose, and throat, spinal fluid, sputum, and wounds. From these cultures the following bacterial types and strains were isolated: *Diplococcus pneumoniae*, *Streptococcus hemolyticus*, and hemolytic *Staphylococcus aureus*. This method proved equally efficient for all organisms.

An attempt to correlate the in vitro experiments with the in vivo effectiveness of the sulfonamides is in progress.

CONCLUSIONS

1. A simple, quick method for testing the inhibiting effects in vitro, of sulfanilamide, sulfapyridine, sodium sulfapyridine, sulfathiazole, sodium sulfathiazole, sulfadiazine, and sodium sulfadiazine upon the growth of various bacterial types and strains is described.

2. The drug of choice can be determined as soon as the control cultures are positive.

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A SIMPLIFIED METHOD FOR FEEDING RATS

H. Ward Ferrill, Ph.D., Chapel Hill, N. C.

A SATISFACTORY method for placing known quantities of solutions directly into the stomach of white rates becomes more and more important when the number of animals is large and laboratory assistants are unavailable. The author has found the following method very satisfactory over a period of several years; it is simple and eliminates practically all danger of injury to the animal.

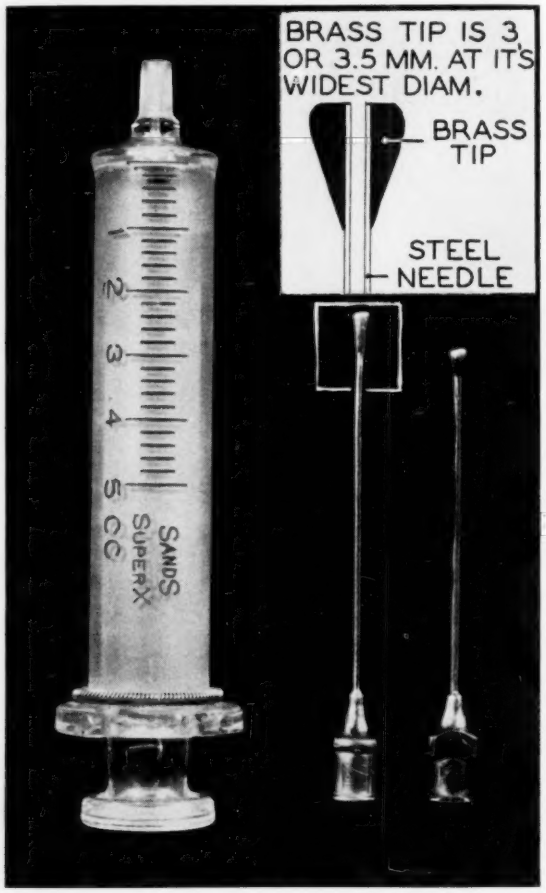

Fig. 1.

An ordinary hypodermic syringe of any desired capacity is used for the accurate measurement of the material. A long, large or small bore needle is used and on its end a small brass knob is soldered. The brass extends far enough up the needle to give it a good area of contact, and an opening the same

*From the Department of Physiology, University of North Carolina School of Medicine. Received for publication, January 29, 1943.

size as the bore of the needle is made through the center of the attached metal. The brass is polished to a very smooth finish so that it can be inserted easily and irritation of the esophagus or cardiac sphincter avoided. It has been found helpful to have different sized needles and knobs for various sized rats if the work is begun when the animals are small. The size which will slip easily down the esophagus of half-grown or adult rats is too large for young animals. The diameters of the knobs used by the author range from 3.0 to 3.5 mm., the smaller being satisfactory for rats of weaning age. A needle at least 2.5 inches in length is more satisfactory than shorter ones.

The animal is held in the left hand with the fingers well forward on the neck to tilt the head back slightly. Then the needle is passed directly down the esophagus into the stomach, and the solution is expelled by pushing in the plunger of the syringe. The animal may clamp the needle with its teeth, but this does not interfere as it does when using a rubber catheter. No gag is necessary for inserting the needle.

Many thousands of injections have been made with this procedure without a single case in which the needle passed into the trachea. The method is very rapid; one person can feed as many as 120 animals per hour after very little training. Autopsies on experimental animals indicate that there is no gross or microscopic change in the esophageal mucosa from daily use of this procedure.

Fig. 1 shows the relative proportions of the various parts of the instrument.

A RAPID METHOD OF PREPARING PNEUMOCOCCAL SPECIFIC CARBOHYDRATES WITH THE AID OF AN ELECTRIC CURRENT*

MARTIN BURGER, M.SC., NEW YORK, N. Y.

THE methods generally used to prepare soluble specific substance of pneumococci[1-3] require autolysis of the cocci. From the autolyzate containing the specific soluble substance and large amounts of protein, the latter is removed. Both the autolysis and the elimination of protein are time-consuming, and we thought it worth-while to investigate a method which would require neither autolysis nor the removal of large amounts of protein.

In 1935, Yen and Kurotchkin[4] and Murao and Morimoto[5] showed that immunologically specific substances could be prepared by electrolyzing suspensions of certain organisms. The former authors obtained the soluble specific polysaccharides of Friedländer's bacillus and *Monilia tropicalis;* and the latter authors obtained a clear filtrate from a suspension of *Bacillus typhosus,* which was said to be more efficient for immunization of mice than a corresponding heat-killed suspension of the same organism.

EXPERIMENTAL

Recent mouse-passed cultures of Types II, III, VI, XII, XIII, XIV, XIX, and XXIII pneumococci were grown for 18 hours in beef-heart broth medium containing 3 per cent of normal horse serum. The organisms were collected

*From the Bureau of Laboratories, Department of Health.
Received for publication, February 17, 1943

by centrifugation and washed twice with 0.85 per cent saline. The average yield of packed cells from ten liters of broth was 15 c.c. The cells were suspended in 150 c.c. of 0.85 per cent NaCl solution, transferred to a 600 c.c. beaker, and layered with 10 c.c. of toluol. A pair of platinum wire electrodes (B. & S. 19 gauge) were placed 6 cm. apart and immersed in the suspension. A variable resistance or bank of lamps in series with the electrodes served to control the voltage across the electrodes. The voltage was maintained between 18 and 22, and the current was 0.45 amperes. A water bath around the beaker was kept between 20° and 22° C., and a thermometer, to note the temperature and at the same time used as a stirrer, was kept in the suspension throughout the electrolysis. The temperature was maintained below 30° C. at all times. The solution was stirred every half-hour in order to remove bacterial protein substances which accumulated on the electrodes. The electrolysis was stopped when the solution became almost transparent. The time required for the dissolution of 15 c.c. of organisms was usually 20 to 24 hours. Most of the precipitated protein which formed was in the toluol layer, and the small amount of suspended matter proved, on microscopic examination, to be bacterial debris and some intact organisms. The latter, however, gave negative Neufeld quellung reactions.

The solution was separated from most of the precipitate by centrifugation, then filtered through filter paper, and the slightly turbid filtrate was further clarified by centrifugation in an angle centrifuge. The clear supernatant was dialyzed for 18 hours against running tap water and finally concentrated in vacuo to one-fifth the original volume of suspension. Any insoluble matter which formed was eliminated by filtration. A small portion of the clear filtrate, when tested for specific polysaccharide, gave an immediate heavy precipitate in homologous antiserum, but not in heterologous antisera. To the main filtrate were added 3 volumes of 95 per cent ethanol. A flocculent precipitate formed, which increased in amount on the addition of 5 c.c. of saturated sodium acetate solution containing 0.5 c.c. of glacial acetic acid. The precipitate, after settling in the refrigerator, was separated by centrifugation, dissolved in 25 c.c. of distilled water, and a 1 c.c. sample tested for the presence of protein. If protein was present (i.e., positive biuret or trichloracetic acid tests), the main solution was shaken with chloroform-butyl alcohol mixture, as recommended by Sevag.[6] Six extractions with the chloroform reagent usually removed all of the protein, as evidenced by negative biuret and trichloracetic acid tests. The polysaccharide was reprecipitated by adding 2 volumes of ethanol, allowed to stand in the refrigerator overnight, and centrifuged the following day. The alcoholic supernatant was discarded, and the precipitate suspended in 95 per cent ethanol, filtered on a Buchner funnel, washed with absolute alcohol, ether, and finally dried in a vacuum desiccator. The average yield of specific carbohydrate from the organisms grown in 10 liters of medium was 125 milligrams.

The substances were type-specific, as no cross reactions were obtained in heterologous antipneumococcal sera. They all gave a positive precipitin reaction in a dilution of $1:10^6$ when tested against homologous rabbit antiserum. In the dry state, the substances were yellowish in color and readily soluble in water. One per cent solutions gave negative Millon, Hopkins-Cole, trichloracetic

acid, ninhydrin, biuret, and xanthoproteic acid tests for protein. The Molisch tests were positive, and the Fehling tests before acid hydrolysis were negative. After acid hydrolysis, the Fehling tests were positive.

A control suspension of organisms of each of the types studied was transferred to a thin-walled test tube without electrodes and kept immersed in the main suspension during the electrolysis. The nondissolution of the control organisms was confirmed by negative precipitin reactions on the supernatant after separation of the organisms by centrifugation.

The effect of different electrolytes on the dissolution of pneumococci with the aid of an electric current was also studied. One per cent solutions of NaCl, $NaNO_3$, Na_2SO_4, or $NaC_2H_3O_2$ were used as the suspending media. Four, 0.5 c.c. portions of packed Type III organisms (Type XIV and Type II organisms were treated similarly) were transferred to tubes, and each sample of cells was washed twice with one of the appropriate electrolytes. The washed cells were then suspended in 30 c.c. of the appropriate electrolyte, layered with 5 c.c. of toluol, subjected to the electric current, and treated as described above. With each electrolyte, dissolution of the organisms was complete at the end of four hours, as evidenced by the complete clearing of the suspension. Microscopic examination of the precipitate which formed showed only bacterial debris present. Chemical examination proved that the precipitate contained protein. One-tenth cubic centimeter portions of the clear aqueous solution gave positive precipitin reactions with homologous antiserum and no reactions with heterologous antisera. Control tubes containing 2 c.c. of the original suspension and not subjected to electrolysis remained milky.

After each suspension had cleared, it was filtered on a wet filter paper to retain the toluol and most of the precipitate. The cloudy filtrate was then passed through a Berkefeld N candle, and the clear filtrate on diluting with two volumes of 95 per cent alcohol gave a precipitate, which, after standing several hours in the refrigerator, was filtered, washed with absolute alcohol, ether, and finally dried in vacuo. No attempt was made to purify the substances obtained in these experiments because of the small amount isolated. One per cent solutions gave positive Molisch tests, faintly positive biuret tests, and negative trichloracetic acid, sulfosalicylic acid, and xanthoproteic acid tests.

The pH of each suspension before and after electrolysis was:

NaCl	– 7.4 before; 8.0 after electrolysis
$NaNO_3$	– 7.3 before; 3.4 after electrolysis
Na_2SO_4	– 7.5 before; 2.8 after electrolysis
$NaC_2H_3O_2$	– 7.7 before; 5.7 after electrolysis

With the exception of the NaCl electrolyte, the pH decreased after electrolysis.

These results may explain the views of Yen and Kurotchkin[4] that the liberation of specific polysaccharide substances during electrolysis in sodium chloride solution is attributable to three factors: (a) The sodium hypochlorite which is formed during electrolysis causes lysis of the organisms; (b) the sodium hydroxide which accumulates combines with bacterial protein; (c) the alkali proteinate is attracted to and precipitated at anode, leaving the specific carbo-

hydrate in solution. In the $NaNO_3$, Na_2SO_4, and $NaC_2H_3O_2$ solutions, probably only (b) and (c) are involved as no sodium hypochlorite is formed.

SUMMARY

A comparatively rapid method of preparing protein-free soluble specific substances of pneumococci is described. The substances obtained may be used in the routine standardization of antipneumococci sera. In our procedure, autolysis is replaced by subjecting a suspension of the organisms in NaCl solution (or in $NaNO_3$, $NaC_2H_3O_2$, or Na_2SO_4 solution) to an electric current. Most of the protein is precipitated during electrolysis. The type-specific capsular polysaccharide is isolated from the aqueous solution of the electrolysate by methods similar to those of Heidelberger, Kendall, and Scherp,[1] and Goebel et al.[2]

I wish to thank Mrs. H. Bucca and Miss A. Berlow for their assistance in the bacteriologic work required in this study.

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VENIPUNCTURE IN THE PRESENCE OF EDEMA*

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CONSIDERABLE difficulty is often encountered in locating a vein for the purpose of venipuncture in the presence of marked edema involving the arms. The most common procedure in these cases has been to cut down on the vein.

The writer wishes to describe a simple method for which no priority is claimed, but which does not seem to be commonly known or employed. The technique is as follows: The patient's elbow is held by the physician in such a manner that the thumb is over the antecubital space. Pressure is exerted by the thumb for a period of from thirty to sixty seconds producing pitting. In the bottom of the resulting "pit" the antecubital veins stand out very prominently, since the edema fluid has been expressed not only from the overlying tissues but from the tissues surrounding the veins as well. So prominent are the veins made by this technique that the use of the tourniquet may occasionally be superfluous.

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SIMPLIFIED KLOTZ-MACLACHLAN SOLUTION FOR TEMPORARY COLOR PRESERVATION*

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AMONG the criteria for the ideal fixing fluid are color preservation, ease of preparation and use, rapid fixation of tissue, low cost, and permanency. It is, of course, difficult to find in a single fluid all these desirable properties. With the advent and popular acceptance of color photography the necessity for permanent preservation of specimens became lessened. A good colored lantern slide is a fine permanent record.

In many cases, where the services of a photographer are not immediately available, the specimen must be preserved for a few hours or days in as near a normal state as possible. At times, the specimen itself may be needed for a single staff conference, student group, or other occasions where a permanent mount is not required but natural appearance is desirable. Here color preservation, ease of preparation, and adequate fixation for future sectioning are the chief considerations.

By use of a suitable fixing fluid the specimen can be photographed, demonstrated, sectioned at leisure, and finally discarded or disposed of otherwise. With such a routine, storage space can be kept to a minimum.

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In our search for a solution to meet the requirements of this routine various combinations of the Klotz-MacLachlan[1] two-solution procedure were investigated. By modifying the concentration of some of the constituents we were able to develop one solution which has been satisfactorily used in a number of laboratories for more than one year. It fulfills the criteria of color preservation, ease of preparation, and adequate fixation. It is not prohibitively expensive.

The solution is made as follows:

Artificial carlsbad salt	487 Gm.
Chloral hydrate	175 Gm.
Formalin (40 per cent)	150 c.c.
Distilled water to make	19,000 c.c. (5 gallons)

The formula for artificial Carlsbad salt is five times the amount given by MacLachlan[2] but is otherwise unchanged. This is so reported for convenience in preparing the solution.

Sodium sulfate	110 Gm.
Sodium bicarbonate	100 Gm.
Sodium chloride	90 Gm.
Potassium nitrate	190 Gm.
Potassium sulfate	10 Gm.

Care must be used to add enough of the solution to the jar. At least three, and preferably five volumes of solution must be used for each volume of tissue. The specimen must be kept submerged, and it is our practice to overlay with cotton batting those which float. The concentration of the formalin must be watched, and if the color fades too rapidly 100 instead of 150 c.c. may be used in the formula, but this results in somewhat less adequate fixation. We have noted that the red colors, such as are seen in a superficial vascular area, are heightened for a few minutes or an hour after contact with the fluid. By photographing specimens with these features within the specified time, striking color photographs may be obtained.

Specimens may be preserved from seven to twenty-one days, and with certain types of tissue even longer. Fatty tissue keeps the least well. Cytologic detail is not quite so good as formalin fixation, especially if the blocks are cut after the first week or adequate fluid has not been used. The simplicity and color-preserving qualities, however, are ideal, and for temporary preservation this solution is well suited.

SUMMARY

A modification of Klotz's fixing fluid is suggested which temporarily preserves color, adequately fixes tissue, and avoids excessive technical procedure.

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USE OF THE SOUTH AFRICAN CLAWED FROG (XENOPUS) AS AN ASSAY ANIMAL FOR GONADOTROPIC HORMONES*

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IN 1930, Hogben[1] first showed that anterior pituitary lobe extracts could cause external deposition of ova in the mature female *Xenopus laevis* (South African clawed frog). Following this original observation, other investigators corroborated Hogben's finding, and the use of the female Xenopus as an assay animal for gonadotropic substances then became established in South Africa.

The Xenopus differs from the common variety of American frogs in that it constantly ovulates and accordingly carries the ova in its abdomen. The eggs are extruded in natural life only upon stimulation by the male of the species. The Xenopus has never been seen to extrude any eggs when kept segregated from males under laboratory conditions, unless injected by hormones. Thus, the animal is ever ready to expel its eggs upon proper stimulation. The external deposition of eggs has come to be accepted as a reliable manifestation of gonadotropic activity.

Having previously shown that egg extrusion could be attained by injection of extracts from the urine and the serum of pregnancy, a comparative study of the efficacy of true anterior lobe extracts seemed to be the next logical procedure. In our previous investigations[2, 3] it was demonstrated that deposition of eggs could be induced equally successfully by 100 rat units of chorionic gonadotropin of pregnancy urine and from 30 Cartland-Nelson units or 1,000 international units of equine gonadotropin derived from pregnant mares' serum. The Xenopus thus affords an excellent opportunity to evaluate the comparative gonadotropic activity of the various hormone extracts.

PROCEDURE

The Xenopus unit was based on the deposition of eggs within 6 to 18 hours after a dorsal subcutaneous injection of the hormone tested. The smallest amount of hormone which constantly caused extrusion was defined as one Xenopus unit. For each separate dosage of hormone to be tested two mature female Xenopus frogs were used. Each Xenopus was placed in a two-gallon tank filled one-third with water. Each tank was furnished with a half-inch wire mesh placed one inch above the bottom of the tank to prevent the possibility of the Xenopus eating any of its eggs.

Sixteen tanks were set up for eight different dosages of the powdered gonodatropic factor of sheep anterior pituitary gland.† Tanks A and A′ held

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frogs injected with 5 rat units; the frogs in B and B′ received 10 rat units; those in C and C′ received injections of 15 rat units; D and D′, 25 rat units; E and E′, 50 rat units; F and F′, 75 rat units; G and G′, 100 rat units; H and H′, 200 rat units.

The animals were all injected through the dorsal lymph space and were kept at room temperature. They were examined periodically up to 18 hours after injection. After 18 hours it was noted that the animals in A, A′, B, B′, C, C′ had not reacted by egg extrusion, while the animals in D and D′ (25 rat units) showed definite evidence of egg deposits. Similarly, the animals in the other tanks that received larger doses also reacted positively.

This entire experiment was repeated the following week with similar results being obtained.

RESULTS

From the data compiled in Table I it will be seen that anterior pituitary gland extracts of sheep are highly gonadotropic in the female Xenopus. Quantitatively, 25 rat units were sufficient to produce external deposits of eggs in these animals. On the basis of this and our previous findings, it must be concluded that even though pregnant mare's serum and pregnancy urine extracts do cause deposition of eggs, true pituitary extracts are more efficacious in producing this reaction and with smaller doses.

TABLE I

MINIMAL AMOUNTS OF GONADOTROPINS WHICH ARE CAPABLE OF INDUCING EGG EXTRUSION IN THE FEMALE XENOPUS LAEVIS

CHORIONIC GONADOTROPINS DERIVED FROM PREGNANCY URINE (2)		CHORIONIC GONADOTROPINS DERIVED FROM PREGNANT MARES' SERUM (3)		GONADOTROPINS DERIVED FROM ANTERIOR PITUITARY LOBE OF SHEEP	
Antuitrin-S	100 rat units[1]	Gonadogen	30 Cartland-Nelson units[3]	Gonadophysin	25 rat units[5]
Follutein	100 international units[2]	Anteron	1000 international units[4]		

[1]Chorionic gonadotropin from human pregnancy urine, Parke, Davis & Co., Detroit, Mich.
[2]Chorionic gonadotropin from human pregnancy urine, E. R. Squibb & Sons, New Brunswick, N. J.
[3]Equine gonadotropin, Upjohn Co., Kalamazoo, Mich.
[4]Equine gonadotropin, Schering Corp., Bloomfield, N. J.
[5]Anterior pituitary lobe extract of sheep, G. D. Searle & Co., Chicago, Ill.

Since the Xenopus was found to be so accurate in evaluating the activity of the gonadotropes, the animal has been utilized clinically in the assay of urine. Urines from normal nonpregnant women, pregnant women, patients in the postmenopausal state have been assayed by means of the Xenopus. A Xenopus unit is equivalent to that amount of gonadotropic substance which will cause external egg extrusion in two Xenopus within 6 to 18 hours. The results of our clinical assays are of such interest that they will be published separately in a subsequent publication.

SUMMARY

Extracts of the anterior pituitary gland of sheep when injected into the South African Clawed Frog (Xenopus) were capable of inducing ovulation and external extrusion of eggs with doses as small as 25 rat units. Simila

gonadotropic activity with other extracts are evaluated. The use of the Xenopus frogs for assaying the urine of patients for gonadotropic substances is suggested.

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CHEMICAL

ESTIMATION OF SERUM GLOBULIN BY MEASUREMENT OF VISCOSITY OF FORMALIN-TREATED SERUM: PROTEIN PARTITION BY PHYSICAL METHODS*

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WITH THE TECHNICAL ASSISTANCE OF MISS MARGARET A. ADAMS, A.B.

INTRODUCTION

SINCE serum proteins are often altered in the course of disease, information concerning their total concentration and the relative proportions of albumin and globulin is important for diagnostic and therapeutic purposes. One of the most widely used methods for obtaining these data is the micro-Kjeldahl chemical determination of nitrogen,[1] in conjunction with the modified Howe's method of separation of albumin and globulin by sodium sulfate.[2] This is a time-consuming and elaborate procedure requiring skilled training.

The falling drop technique devised by Kagan,[3, 4] employing physical methods, has simplified the determination of the total protein of the serum. However, no methods comparable in simplicity for measuring the fractions of albumin or globulin appear to be available. The combined chemical and physical method advanced by Barbour[5] for albumin estimation is somewhat complicated; the usual tests for globulin such as the Takata-Ara, formol-gel, etc., are qualitative and indicate only the presence of hyperglobulinemia.

In a previous study[6] a modification of the formol-gel reaction was described for estimating the globulin content of sera in hyperglobulinemia. Confirming Napier's work, which demonstrated the specificity of the formol-gel test for globulin, it was observed that for a 5 per cent purified human albumin solution, the addition of small amounts of formaldehyde caused no change in viscosity. With reference to the blood serum, it was shown by direct measurements that the addition of formaldehyde to the serum increases its viscosity and that the degree of change corresponds to the concentration of globulin.

The modified formol-gel reaction[6] is employed in sera containing abnormally increased concentrations of globulin, which can be estimated quantitatively by the observation of a readily detected change in viscosity. This is determined on samples of serum placed in test tubes and then diluted progressively with physiologic saline and treated with formaldehyde. After standing for a period of twenty-four hours at room temperature, the tubes are shaken vigorously, and a degree of viscosity is noticed in one of the dilutions in which the air bubbles produced by the shaking do not rise to the surface, but remain

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stationary, i. e., the "stationary bubble" end point. In less viscous dilutions the bubbles rise slowly, in more viscous dilutions gelification occurs. The "stationary bubble" end point corresponds to a globulin concentration of 3.1 Gm. per 100 c.c. as determined by chemical methods. From such observations the globulin content of a given serum may be calculated by the formula: Gm. per 100 c.c. globulin $= \frac{3.1}{y}$, where y represents the fraction of 1.0 c.c. of serum present in the dilution showing the above end point. Accordingly this method employs an indirect measurement of viscosity and is limited in its uses to cases of hyperglobulinemia.

In the present study the method has been extended to include all ranges of concentration of serum globulin. Direct measurements of the viscosity of formalin-treated sera were correlated with their globulin content. From these data a formula was derived for the determination of all ranges of concentrations of serum globulin. The final object was to estimate the total protein of the blood serum by the falling drop technique, the globulin by the measurement of the viscosity of formalin-treated serum, and the albumin by subtraction of one from the other. The results obtained by physical and chemical methods were compared.

PART I

DEVELOPMENT OF A FORMULA FOR THE CALCULATION OF THE GLOBULIN CONTENT FROM THE MEASUREMENT OF VISCOSITY OF FORMALIN-TREATED SERUM

A. *Viscosity Measurements on Serums With Hyperglobulinemia.*—Twenty samples of venous blood were taken during the fasting state from 19 patients with hyperglobulinemia. The blood was allowed to clot and the serum drawn off. For each serum a series of tubes was set up, each containing an accurately measured amount of serum ranging from 0.20 c.c. to 1.92 c.c. respectively and then diluted with physiologic saline to a final volume of 2.00 c.c. To each dilution was added 0.1 c.c. U.S.P. formaldehyde. After shaking, the tubes were allowed to stand at room temperature for twenty-four hours.

Depending upon their ability to flow easily through the viscosimeter, two to five dilutions from a series were chosen for direct observation of viscosity with reference to physiologic saline as unity. The viscosity was measured by observing the rate of flow of each dilution in an Ostwald pipette[7] of 2 c.c. capacity immersed in a water bath at a constant temperature of 25°C. The Ostwald pipette was allowed to stand in the water bath for ten minutes in order to reach 25° C. before the rate of flow was measured, and each measurement was repeated 3 times to obtain an average. The viscosity was determined by the formula $V = \frac{T_1 \times S.G.}{T_2}$. Where V = viscosity; T_1 = time of flow in seconds of formalin-treated dilution; T_2 = time of flow in seconds of physiologic saline; and S.G. = specific gravity of formalin-treated dilution. Since the specific gravity need be expressed to only two significant figures this factor becomes 1.0. A total of 66 dilutions were studied for their viscosity.

B. *Globulin Determinations.*—The globulin content of each serum in which the viscosity was measured in the above manner was determined in two ways:

(1) by the "stationary bubble" end point of the modified formol-gel reaction, and (2) by micro-Kjeldahl nitrogen analysis in conjunction with the modified Howe's method of separation of albumin and globulin. The globulin values, obtained by means of the "stationary bubble" end point, compare closely with the values found chemically with an average difference of 5 per cent and individual variations from 0 to 15 per cent (Table I). Since the results obtained by the "stationary bubble" end point could be reproduced more consistently, they were selected as the standard to calculate the globulin concentration of the dilutions prepared from each serum.

TABLE I

GLOBULIN VALUES, GM. PER 100 C.C. DETERMINED BY TWO METHODS

RESULTS	MODIFIED FORMOL-GEL REACTION "STATIONARY BUBBLE" END POINT	CHEMICAL $NaSO_4$ PRECIPITATION AND MICRO-KJELDAHL
1	3.5	3.49
2	3.9	3.99
3	3.7	3.74
4	4.3	3.80
	No. 4 repeated 2 days later	
5	3.9	3.66
6	3.8	3.75
7	4.0	4.18
8	3.1	3.13
9	5.7	5.87
10	4.8	5.20
11	4.4	4.06
12	4.3	4.04
13	3.7	4.03
14	4.0	4.05
15	3.7	3.57
16	3.1	3.63
	No. 16 repeated 2 days later	
17	3.1	3.25
18	3.1	3.37
19	3.4	3.43
20	4.1	4.28

C. *Derivation of Formula Expressing Globulin Concentration in Terms of Viscosity.*—The viscosity values of the 66 dilutions mentioned in Section A were related to their respective globulin values mentioned in Section B. By plotting the globulin values against the log. (viscosity — 1), a straight line was obtained when the globulin concentration of the dilutions was less than 2.5 Gm. per 100 c.c. Above this level the straight line did not persist. The equation of the straight line, determined by the customary method of least squares, was found to be $G = 1.55 \log. (V-1) + 1.64$ where G is the grams of globulin per 100 c.c. and V is the viscosity. Fig. 1 shows all the data and the straight line in the region to which it may be applied.

PART II

APPLICATION OF THE FORMULA $G = 1.55 \log. (V-1) + 1.64$ TO THE DETERMINATION OF ALL RANGES OF GLOBULIN IN BLOOD SERUM

Serum was obtained as before and two test tubes were prepared, one containing 2 c.c. of undiluted serum and the other, 1 c.c. of serum diluted with

1 c.c. of physiologic saline. To each was added 0.1 c.c. U.S.P. formaldehyde. The tubes were shaken, and, after a period of 24 hours at room temperature, observations of viscosity were performed as described above. The undiluted serum was used if it was capable of flowing freely through the Ostwald pipette. Otherwise the diluted serum was used. The viscosity reading was substituted in the formula $G = 1.55 \log. (V-1) + 1.64$, and the globulin concentration multiplied by 2 when serum diluted 50 per cent was used. The total protein in each serum was determined by the falling drop technique of Kagan, and the albumin content was obtained by subtracting globulin values from the total protein.

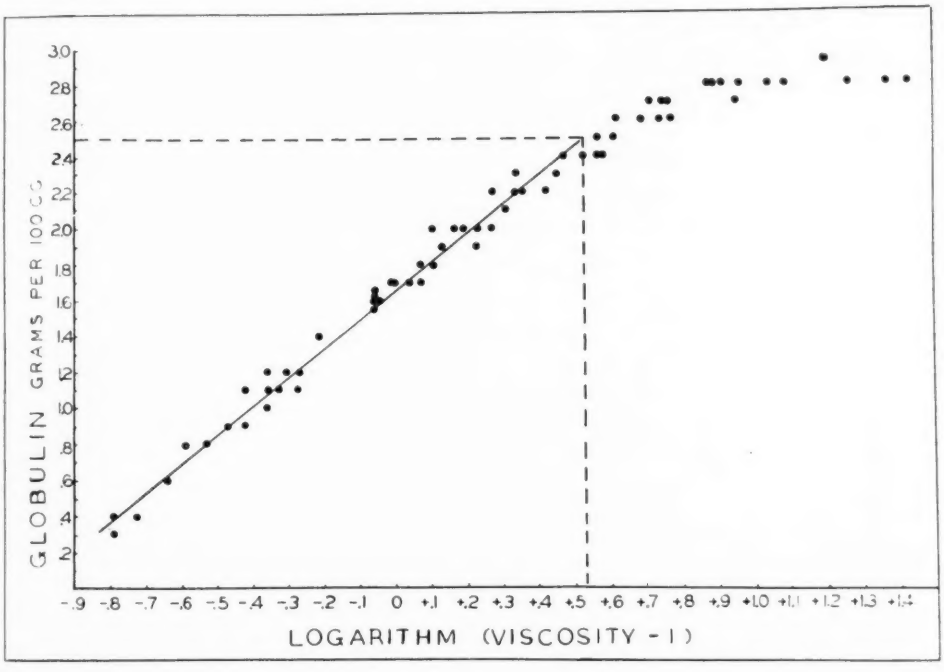

Fig. 1.

The total protein and the globulin and albumin content of 18 sera were determined. The comparative results between the chemical methods and the combined use of the falling drop and the viscosimetric methods are shown in Table II. The differences in globulin content between the chemical and the physical methods varied from 0 to 0.6 Gm. per 100 c.c. with an average of 0.2 Gm. per 100 c.c. and an average percentage difference of 7; the variations in the case of albumin were from 0 to 0.8 Gm. per 100 c.c. with an average difference of 0.2 Gm. per 100 c.c. and a percentage difference of 8. The total protein differences were from 0 to 0.4 Gm. per 100 c.c. with an average of 0.2 Gm. per 100 c.c. and a percentage difference of 4.

Experiments 11, 12, and 13 showed a variation not consistent with the general average. A search for the cause of such irregularity revealed that improper cleansing of the pipettes and especially of the Ostwald pipette was responsible for the abnormal results. When repeated with adequate precautions, the observations were consistent with the earlier determinations.

TABLE II

PROTEIN DETERMINATIONS. COMPARISON BETWEEN PHYSICAL AND CHEMICAL METHODS

		GM. PER 100 C.C.		DIFFERENCES	
		FALLING DROP AND VISCOSIMETRIC METHODS	CHEMICAL METHODS	GM. PER 100 C.C.	PER CENT
1	T.P.	6.5	6.5	0	0
	Glob.	1.9	1.8	+0.1	6
	Alb.	4.6	4.7	-0.1	2
2		6.7	6.4	+0.3	5
		2.0	1.7	+0.3	18
		4.7	4.7	0	0
3		5.4	5.2	+0.2	4
		3.1	3.1	0	0
		2.3	2.1	+0.2	10
4		7.3	7.1	+0.2	3
		2.7	2.6	+0.1	4
		4.6	4.5	+0.1	2
5		5.6	5.4	+0.2	4
		3.5	3.5	0	0
		2.1	1.9	+0.2	10
6		6.0	5.6	+0.4	7
		1.8	1.9	-0.1	5
		4.2	3.7	+0.5	13
7		5.9	5.7	+0.2	4
		3.1	3.4	-0.3	9
		2.8	2.3	+0.5	22
8		5.2	5.2	0	0
		1.7	1.7	0	0
		3.5	3.5	0	0
9		6.4	6.5	-0.1	2
		2.9	3.0	-0.1	3
		3.5	3.5	0	0
10		5.2	5.3	-0.1	2
		2.1	2.3	-0.2	9
		3.1	3.0	+0.1	3
11		6.9	7.2	-0.3	4
		3.0	2.5	+0.5	20
		3.9	4.7	-0.8	17
12		5.6	5.3	+0.3	6
		3.6	3.0	+0.6	20
		2.0	2.3	-0.3	13
13		6.0	5.7	+0.3	6
		4.5	4.1	+0.4	10
		1.5	1.6	-0.1	6
14		7.0	6.6	+0.4	6
		3.0	3.0	0	0
		4.0	3.6	+0.4	11
15		5.6	5.6	0	0
		2.7	2.9	-0.2	7
		2.9	2.7	+0.2	7
16		8.2	7.8	+0.4	5
		4.1	3.9	+0.2	5
		4.1	3.9	+0.2	5
17		6.2	5.9	+0.3	5
		3.9	3.9	0	0
		2.3	2.0	+0.3	15
18		7.2	7.0	+0.2	3
		3.4	3.3	+0.1	3
		3.8	3.7	+0.1	3

CONCLUSIONS

1. The addition of small amounts of formaldehyde to blood serum produces an increase in the viscosity of the serum. This change is due to globulin, since it does not occur when formaldehyde is added to a 5 per cent solution of purified human albumin.

2. A strictly linear relation was demonstrated between the degree of viscosity and the globulin content of formaldehyde-treated serum, when the globulin content was below 2.5 Gm. per 100 c.c. This relation is expressed by the equation $G = 1.55 \log. (V-1) + 1.64$ where G = grams of globulin per 100 c.c. and V = viscosity.

3. With this formula it is possible to estimate quantitatively the globulin concentration of the blood serum by measuring its viscosity after the addition of formaldehyde.

4. The value of the viscosimetric method using formaldehyde-treated serum for the estimation of globulin is enhanced when used together with the falling drop method for estimation of total protein. Through their combination the total protein, albumin, and globulin content of any serum can be estimated entirely by physical means with great saving of time, material, and number of personnel.

5. A comparison of the physical and chemical methods shows an average difference of 0.2 Gm. per 100 c.c. for total protein, albumin, and globulin. This error is similar to that inherent to the chemical method.

6. The careful observance of certain precautions must be emphasized: the equipment must be kept meticulously clean; the amounts of serum, saline, and formaldehyde should be measured with accuracy; the water bath must be kept at a constant temperature.

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A METHOD FOR EXACT PROTEIN DETERMINATION IN SMALL QUANTITIES OF SPINAL FLUID*

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ALTHOUGH the knowledge of the exact protein content of the spinal fluid is frequently of diagnostic importance, the present methods when carried out with small quantities of fluid yield results which may show an error as high as 100 per cent.[1] Exact results may, of course, be obtained with the old Kjeldahl method, but this requires such large quantities of fluid that they cannot as a rule be obtained, or only with difficulty.

PRINCIPLE OF THE NEW METHOD

The total proteins of the spinal fluid are precipitated with phosphomolybdic acid, centrifugated, and the sediment, after being dissolved in diluted sodium hydroxide, is quantitatively carried over to a Kjeldahl flask. The further procedures are those of nonprotein nitrogen determinations.[2, 3]

REAGENTS

1. For protein precipitation:

(a) Dissolve 5 Gm. of anhydrous sodium sulfate and 8 Gm. of phosphomolybdic acid in 200 c.c. distilled water.

Add 20 c.c. of 5N sodium hydroxide, and boil the mixture for one-half hour. After cooling, add 10.6 c.c. of concentrated sulfuric acid and water up to 1000 c.c.

(b) A 4 per cent solution of chemically pure sodium hydroxide.

2. For digestion:

Dissolve 1 Gm. of phosphomolybdic acid in about 50 c.c. of water. Add 1 c.c. of Kjeldahl hydroxide and boil for twenty minutes to drive off the possibly present ammonia; add 30 c.c. of concentrated sulfuric acid and 5 c.c. of syrupy phosphoric acid. Add water to make up 100 c.c.

3. For neutralization:

(a) A 27 per cent solution of chemically pure sodium hydroxide.

(b) Dissolve 15 mg. of methyl red and 60 mg. of thymol blue in 20 c.c. of N/1 NaOH and add water to 1000 c.c.

(c) A saturated (about 5 per cent) solution of sodium fluoride.

To make this reagent (3) mix 6 parts of solution a with 1 part of solution b and 3 parts of solution c. This mixture keeps indefinitely.

4. For titration:

(a) Buffer solution. Dissolve 84.5 Gm. boric acid and 15.6 Gm. sodium hydroxide in about 800 c.c. water. Boil vigorously for thirty minutes to remove NH_3. Cool and add water to 1000 c.c.

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(b) Dissolve 20 Gm. of sodium bromide in a little water in a measuring flask. Add 8 Gm. elemental bromine and water to 1000 c.c. Just before using pour 10 c.c. of solution (b) into a 50 c.c. measuring flask. Add 25 c.c. of buffer solution and water to 50 c.c.

(c) Potassium iodide in granules.

(d) Fuming hydrochloric acid diluted with an equal quantity of water.

(e) 0.25 per cent starch solution.

(f) N/100 solution of sodium thiosulfate.

PROCEDURE

Place in a small 5 by 50 m.m. test tube (Widal tubes are best) exactly 1 c.c. of the spinal fluid. If the Pandy reaction is positive, only 0.5 c.c. of fluid is used. Add 2 c.c. of phosphomolybdic acid reagent (1a) to precipitate the proteins. Place in water bath at 56° C. for a few minutes; then centrifuge for about ten minutes at 3,000 revolutions. Carefully pour off the supernatant fluid and dissolve the sediment in 0.3 to 0.5 c.c. of 4 per cent sodium hydroxide (1b). Draw up the contents of the tube with great care, using a serologic 1 $cm._3$ pipette; and place them into a Kjeldahl flask which contains 2 c.c. of the digestion mixture reagent (2) and a few glass pearls. Again fill the tube with some water, and with the same serologic pipette transfer it to the Kjeldahl flask. Repeat this a number of times drawing up and blowing out the contents of the pipette and washing the sides of the tube. Set up two Kjeldahl flasks with the fluid to be examined and two flasks filled only with the phosphomolybdic acid reagent as blanks. Boil both series with the digestion reagent until the contents of the flasks become colorless and fumes no longer arise. After cooling the flasks, dilute the contents of each with 2 to 3 c.c. of water, and while continuously cooling with water, add, drop by drop, the mixed indicator, NaOH, sodium fluoride solution (3) until it turns blue: all flasks require about the same number of drops. Neutralize, therefore, all the flasks in the series in this manner; then add to each 5 c.c. of the hypobromine solution (Reagent 4b mixed with buffer solution 4A for use). Then add to each a few granules of potassium iodide (4c) and 2 to 3 c.c. of diluted hydrochloric acid (4d). Titrate with N/100 thiosulfate (4f) solution until the mixture is straw yellow. Add starch and continue titration until the colorless end point is reached.

Note: We use for our determinations micro-Kjeldahl flasks whose dimensions are: length of flask, 135 mm.; diameter of neck, 20 mm.; length of neck, 85 mm.; diameter of body of flask, 40 mm.; volume of flask, about 35 c.c.

CALCULATION

1 c.c. of N/100 thiosulfate equals 0.046 mg. nitrogen, which multiplied by the protein number 6.25 corresponds to 0.2875 mg. of protein. This figure multiplied by 100 gives the protein content as mg. per 100 c.c. Example: 2.1 c.c. thiosulfate for 1 c.c. of spinal fluid was used. Calculation: $2.1 \times 0.2875 \times 100 = 60.375$ mg. protein per 100 c.c. of fluid.

The quantity of thiosulfate used is calculated by subtracting the quantity used in the unknown from that in the blank.

The application of this method for the determination of albumin, globulin, and nonprotein nitrogen in spinal fluid will be discussed in another communica-

tion. We shall also report our findings on the protein content of normal spinal fluid as determined by this method.

CONCLUSION

A method is described by which exact protein determinations are possible even in very small quantities of spinal fluid.

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A HISTOCHEMICAL METHOD FOR DEMONSTRATING THE PRESENCE OF SULFONAMIDES IN THE TISSUES*

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A RECENT communication[1] reported the development of new vehicles ("Penetrasols"), which increased the penetration of a large variety of substances through grossly intact skin (for example, "protein" allergens, iron, mercury, bismuth, and the sulfonamides). The penetration of externally applied sulfonamides was demonstrated by the presence of these drugs in the blood and urine. Further evidence of the penetration of sulfonamides into and through the skin, was adduced by biopsy of the skin area and the use of a newly developed specific histochemical color reaction with the sulfonamide.

We report here the details of our method, which we believe may find many other applications. Preliminary report has appeared.[2]

The vehicles studied were of different compositions, but in each instance equal quantities of the vehicle, containing equimolecular concentrations of the drug, were gently rubbed in, in identical manner to skin areas of identical size. The only preceding preparation consisted of clipping the hairs of the guinea pigs as short as possible with a sharp pair of scissors.

General ether anesthesia was used in the guinea pigs; and local anesthesia with procaine in man. A type of block anesthesia was employed through injection of the anesthetic around and well away from the site of excision to eliminate all possible procaine contamination of the excised tissue. This is an indispensable precaution, as the presence of procaine, or even of injected water, could produce erroneous results. The line of excision must be kept at a considerable distance from the site of inunction to avoid displacement of the inunction material with the scalpel. Each excision was performed after a given time had elapsed following inunction.

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PROCEDURE OF TISSUE EXAMINATION

A. *Fixation.*—No liquid fixatives could be used to obtain a *histotopical* demonstration, i.e., a demonstration of the drug's localization and distribution within tissue, because it was evident that significant quantities of sulfonamide might be removed or displaced through transportation with the liquids. To overcome these difficulties, we used a gaseous, instead of a liquid, fixative.* Dry formaldehyde gas was chosen because the use of osmic acid fumes would have obscured our findings. Fixation was carried out as follows: Trioxymethylene (paraformaldehyde) powder is spread in about 1 cm. depth over the bottom of small glass beakers (1 to 4) which are placed within a glass jar and their bases surrounded by 1 cm in depth of powdered trioxymethylene. The jar must be equipped with a well-fitting glass cover (an ordinary butter jar, $4 \times 4 \times 3$ inches, will do). The small beakers are roofed, either with a piece of gauze fixed by a rubber band, or with a small glass lattice. The biopsy specimens are placed flatly on these "sieves." Each beaker is clearly marked to keep a record of the specimens. After the small beakers with their fresh specimens have been placed within the larger jar, the latter is covered and kept closed throughout the experiment by several windings of adhesive tape around the line of closure.

The jars are kept at room temperature and fixation takes place within two to twenty-four hours, depending on the size and thickness of the specimens. The most favorable moment for microtome cutting is immediately after fixation, because some shrinkage and brownish discoloration gradually occur; but, when necessary, satisfactory sections can be obtained even after ten to fourteen days.

B. *Cutting of Sections.*—After fixation, frozen sections of 10 to 20 μ are cut. As our particular purposes necessitated comparable specimens, the sections used were all made of approximately equal thickness (about 16 μ).

In the study of skin penetration, as already stressed by Fuerbringer[3] and later on by others (Zwick, Eller and Wolff, and Duemling[4]), the specimen must be kept in such a position on the freezing table that the epidermal surface is kept toward the operator, never directed toward the knife. This tends to obviate the possibility of carrying material from the epidermal surface into the deeper structures with the movement of the knife. In cutting thin and hairy animal skin, the epidermal part often tends to separate as a thin lamella. This can be overcome by placing the specimen sagittally, at an angle of about 90 degrees to the edge of the knife. Here again, the epidermal surface must be turned away from the knife while cutting.

C. *Chemical Process and Reagents Used.*—The demonstration of sulfonamide within the tissue is based on the formation of a characteristic precipitation. Identification by means of formation of sulfonamide crystals, as carried out with a solution of iodine and sodium iodide, etc., in microchemical analysis[5] is not feasible in histotopical studies because the drug would be displaced from its original site. Similarly Marshall's reagent, commonly used for quantitative sulfonamide analysis, cannot be employed because extraction would occur with the diazotation and with the reaction.

"Ehrlich's Reagent," p-dimethylaminobenzaldehyde, as used by Werner and others[6, 7, 8] for quantitative sulfonamide analysis in blood, urine, and

*For the same reasons we found these technical precautions necessary in other histotopical examinations; i.e., in our studies on the penetration of Fe., Bi., Hg, etc., through the skin.

other biologic fluids, proved most suitable for our purposes. Extensive preliminary trials in vitro and in tissue sections, with solutions of this reagent in water containing various concentrations of mineral acids in acid alcohol, etc., have shown optimal precipitation with the following solution:

p-dimethylaminobenzaldehyde	1.0 Gm.
Alcohol abs.	95.0 c.c.
Concentrated HCl	5.0 c.c.

The purest obtainable aldehyde, having little, or at most a light buff color when dry, is used. The solution is kept in an amber-colored flask, well closed by a ground glass stopper. The liquid gradually becomes yellowish, and it is preferable to use fresh solutions (never more than 2- to 3-weeks-old).

This reagent yields a lemon-yellow precipitate with low concentrations of free sulfonamides and an orange shade with higher concentrations of the drugs. The reacting group of the sulfonamides is their free "aniline," NH_2 group. Because the colored reaction product bleaches out and gradually assumes other shades on exposure to the air, in vitro as well as in the tissue, the sections cannot be made to keep the characteristic color for long. Extensive studies attempting to defer the fading time and to avoid any procedures or admixtures which might tend to produce variation in color have resulted in the adoption of the following routine technique which we have carried out satisfactorily for the past two years on a great number of specimens of human and animal skin.

The sections (16 μ), obtained as described above, are placed *directly* on a clean slide, with a dry brush (camels' hair). Two drops of the p-dimethylaminobenzaldehyde solution in acid alcohol (see above) are dropped on the section. After three to five minutes, 1 to 2 drops of absolute alcohol containing 5 per cent of concentrated HCl are added (1 drop for the smaller and 2 drops for the larger sections). The sections are dried immediately by careful absorption of excess fluid with small pieces of filter paper, or before a rotating fan, or by both methods. (Careful studies under controlled conditions ruled out the possibility that the removal of the acid-alcohol with filter paper could cause displacement of the colored product in the section.) Warming is not advisable. As soon as dry, the sections are covered with the following concentration of dissolved Damar resin: Damar resin 10.0 Gm., Xylene 10.0 c.c. (This "thick" mounting medium has proved superior to Canada balsam, cedar oil, origanum oil, albumen-glycerin, etc., which either extract the colored product, or produce color changes, or lead to quick fading.) Then the cover slip is sealed with liquid paraffin melting at 56° C. Because exposure to air impairs the intensity of the color, after the addition of the acid alcohol the whole procedure must be accomplished as quickly as possible and air bubbles must be removed from under the cover slip. The successive use of acid alcohol and the solution of Damar resin in xylene has the additional advantage of producing a satisfactory and rapid clearing of the sections. Microscopic examination must be as early as possible; and colored photos, if desired, should be taken not later than three to four hours after the reaction has occurred.

As stated, the presence of sulfonamide is characterized by a bright orange or lemon-yellow color. Counterstaining has not proved valuable because the reaction color of the sulfonamide tends to be obscured. Black and white photo-

graphs may show the sulfonamide picture and distribution when the tissues themselves do not contain pigment or other material darker in shade than the orange-yellow sulfonamide reaction.

Figs. 1, 2, 3, and 4 are black and white photographs which were retaken from colored diapositives and which show the different routes of penetration of sulfonamides through the skin of *the same* albino guinea pig after inunction of sulfonamide under strictly comparable conditions, but dissolved in 3 different types of vehicles. The photographs are presented here merely to show the technical possibilities of our method in differentiating various types of distribution in the tissues. (Details on composition of the different vehicles and mode of application, etc., will be the subject of forthcoming communications.)

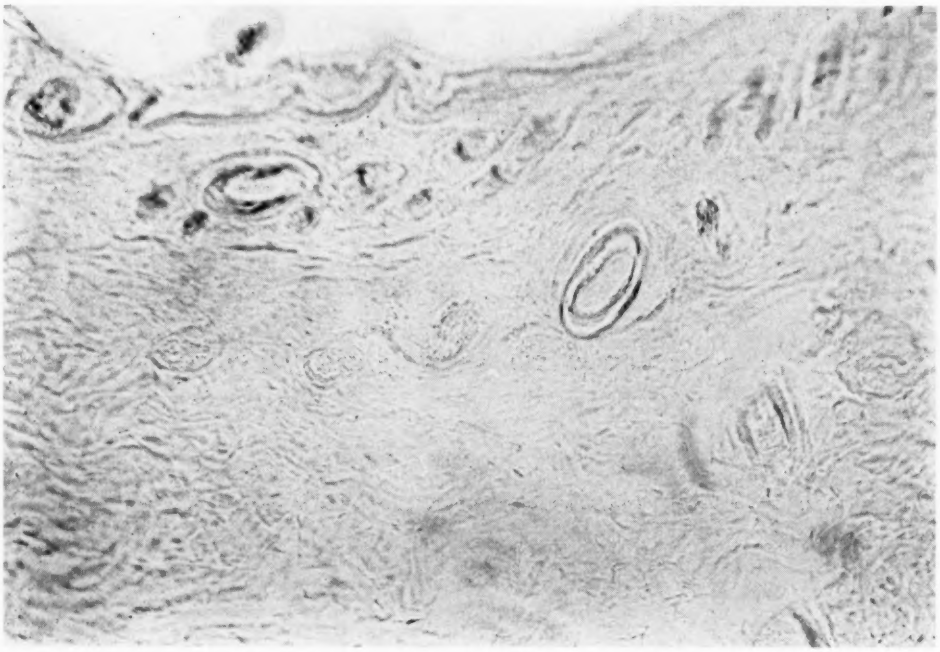

Fig. 1.—Control: Area of skin which received no preceding inunction but which was excised and histologically prepared and treated with p-dimethylaminobenzaldehyde in acid-alcohol exactly like the other sections shown in Figs. 2 to 4. No reaction seen.

Figs. 1 to 4.—Figures are all of specimens of biopsies taken from different skin areas of the same albino guinea pig. (Black and white reproductions of colored diapositives). (Particulars on the composition of the penetrating vehicles designed to achieve the different effects will appear in a future publication.)

D. *Controls.*—Several controls are essential to make as certain as possible that the yellow to orange color observed in the sections is actually due to a sulfonamide reaction and not to the presence of preformed yellowish material or pigments (e.g., melanin, blood pigments, carotene, bile, etc.).

Control 1: Examination of several sections from the same tissue specimen processed in identical fashion as the sections treated with p-dimethylaminobenzaldehyde, but *not* exposed to this reagent. Comparative examination of these sections either shows that preformed colored matter is absent, or reveals the site of any preformed colored matter of a shade similar to that of the sulfonamide reaction product. As a rule, skin sections show little or no mate-

rials in the color range of the sulfonamide reaction; and, if present, their color is duller and more brownish than that of the specific sulfonamide product, which, as stated, is characterized by its brightness.

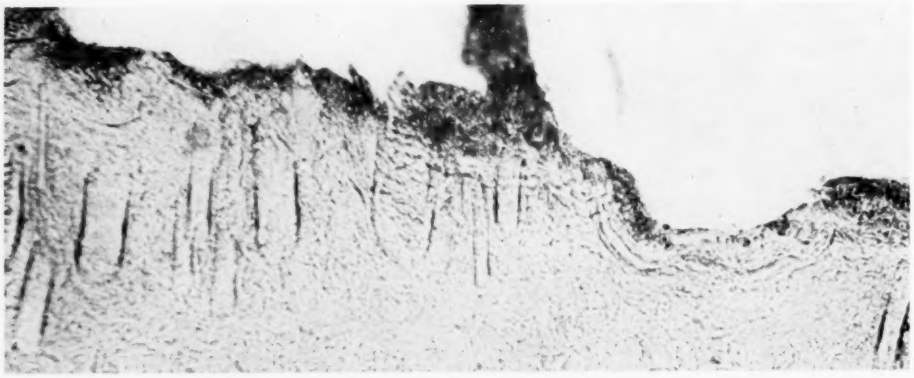

Fig. 2.—Area of skin which received inunction of 10 per cent sulfonamide, applied in a vehicle consisting of lanolin and water. (Excision sixty minutes after inunction; fixation and treatment as described in text.) Note that the reaction product (darker areas) is practically confined to the surface and the upper parts of the horny layer.

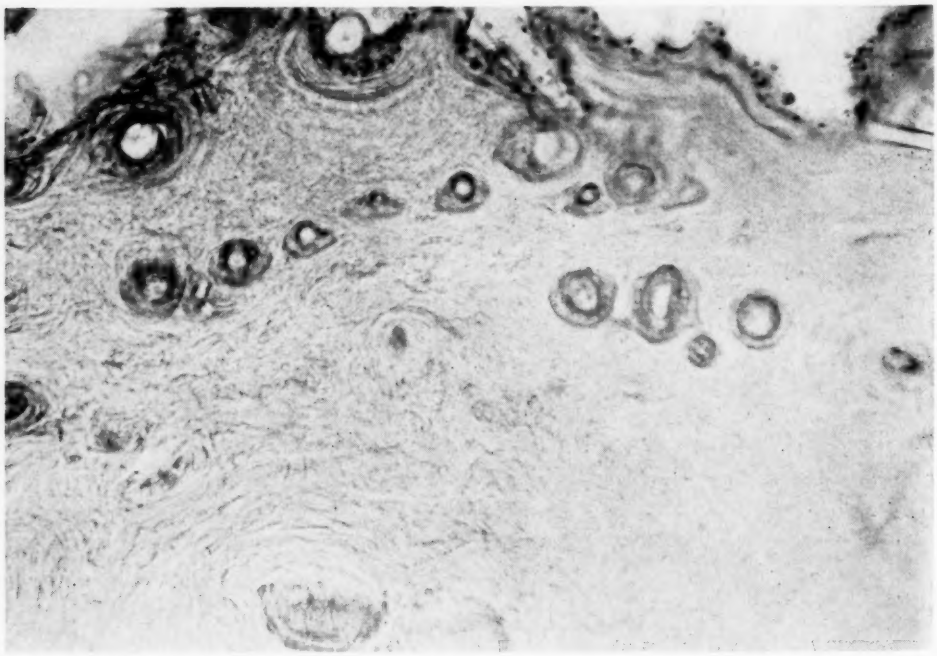

Fig. 3.—Area of skin which received inunction of 10 per cent sulfonamide dissolved in a penetrasol type of vehicle which favors penetration into horny structures. (Excision sixty minutes after inunction; fixation and treatment as described in text.)

(Note that the horny layer and the follicle cross sections show heavy deposits of the dark material, while other parts are relatively free.)

Control 2: Comparative examination of a specimen of the same or corresponding organ but obtained from a site known to be *free of sulfonamide*. Control biopsy specimens taken from the same individual at a skin area distant from that of the sulfonamide application, are fixed, cut, and treated with

p-dimethylaminobenzaldehyde, exactly as described for the demonstration of sulfonamides in the tissue. This is necessary to rule out the possibility of a "nonspecific" reaction. While these controls were uniformly negative (Fig. 1), it must be borne in mind that p-dimethylaminobenzaldehyde produces a yellow reaction with any primary aromatic amine, with urobilinogen, with higher concentrations of urea, and perhaps also with decomposition products of the tissue. We have sometimes observed this phenomena on merely warming a section. (Thus, as stated, warming must not be used in the drying procedure.) Furthermore, certain drugs other than sulfonamides (e.g., phenacetin, acetanilid) give reactions similar to those produced with sulfonamides. Procaine and related local anesthetics may also produce this effect; therefore, as already mentioned, no such solutions should be used to infiltrate the actual site of biopsy.

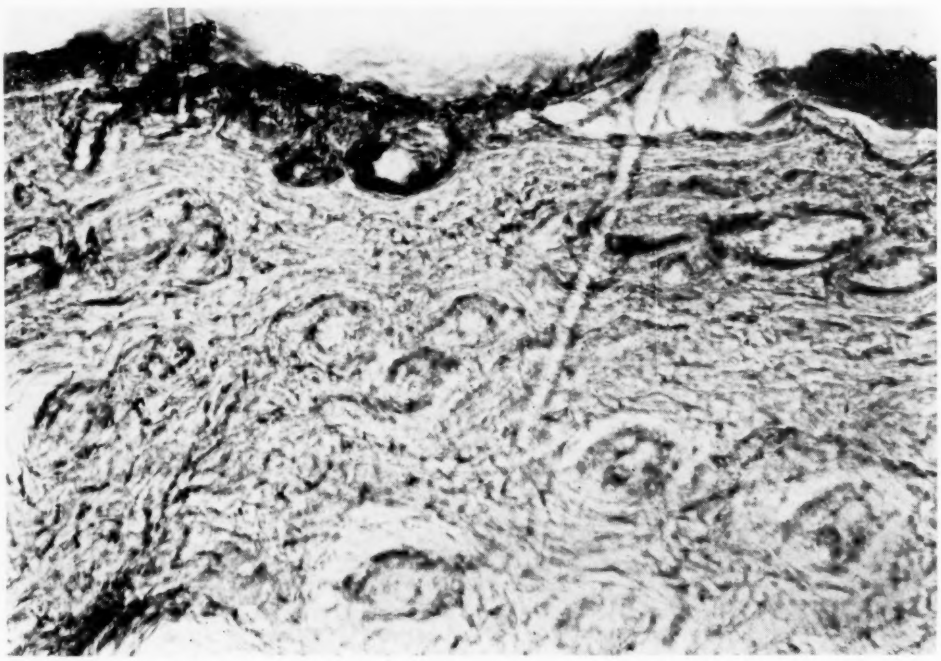

Fig. 4.—Area of skin which received inunction of 10 per cent sulfonamide in a penetrasol type of vehicle which favors penetration of all cutaneous structures. (Excision sixty minutes after inunction; fixation and treatment as described in text.)

(Note that the reaction product [dark material] is found in all structures, including cutis.)

When all precautions have been rigorously observed, the described histotopical method is probably applicable to a large variety of tissues (urethra, other mucous membranes, tonsils, bone marrow, wounds, etc.), but is not likely to prove applicable to certain other tissues (liver, gall bladder, etc.). As is evident, the investigator should not rely upon an *absolute* accuracy of localization; however, when identical experiments *repeatedly* show the *same* localizations of the characteristic colored material; and, conversely, when deliberate experimental modifications *repeatedly* show characteristic *differences* in the localization of the colored material, the findings may be considered as elucidating

particulars of the drug's penetration, route, and distribution within the tissue structures. (See Figs. 2, 3, and 4.)

E. *Discussion.*—A few general comments should be made on our histochemical procedure itself and how we came to adopt the various steps finally selected.

As shown by Morris in reporting on sulfonamide determination in body fluids,[8] the compound responsible for the color is p-dimethylaminobenzylidene-p-aminobenzene sulfonamide. While Morris rightly stressed the dependence of the color on the pH, addition of a buffer is not necessary in our procedure, which is carried out almost in the absence of water. The HCl concentration used is so high that variations which may be caused by pH differences in the specimens are negligible.

In order to minimize the possibility of dissolving or moving parts of the reaction product, our sections are not immersed in the reagent solution, but are treated with the reagent directly while on the slide. On the other hand, the plain acid (HCl)-alcohol is added as a rinse, to ensure the removal of excess reagent and of other loose yellow or orange-colored matter. This rinsing unavoidably involves the loss of a fraction of the reaction product, but we believe this disadvantage to be more than offset by the advantage of confining the colored reaction product as strictly as possible to the original actual sites of the drug within the tissues. Our results are, therefore, more inclined to miss some of the sulfonamide, rather than to indicate sulfonamide where none was present in the tissues.

As already pointed out, the characteristic picture is not preserved for long, despite all available technical precautions to prevent fading. While undeniably inconvenient, the lack of stability of the color is further proof of the nature of the colored product and indicates that the yellow or orange material is produced by the chemical reaction with the sulfonamide and that it is not due to the presence of other or preformed colored matter, such as pigment, lipids, carotene, etc.

SUMMARY

A new method for histochemical and histotopical identification of sulfonamides in tissue sections is described and discussed. This method has been employed and found satisfactory in numerous studies on the percutaneous penetration of sulfonamides, after their solution in penetrating vehicles and their external application to grossly intact human and animal skins.

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THE DETERMINATION OF ALBUMIN AND GLOBULIN IN BLOOD SERUM BY SPECIFIC GRAVITY MEASUREMENTS*

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SPECIFIC gravity methods[1-4] suitable for the determination of total protein in serum may be applied to the estimation of the albumin and globulin fractions. In a procedure described by Barbour,[5] serum is mixed with an exactly equal volume of saturated ammonium sulfate under oil and is spun in an angle centrifuge for an hour at high speed. The density difference between the supernatant liquid and a like mixture, in which serum is replaced by an accurately standardized dilute solution of ammonium sulfate, gives the basis for calculating the amount of albumin present. Certain rigorous precautions are necessary. On account of the great difference in the specific gravities of serum and saturated ammonium sulfate, a small error in measuring the volume of either solution makes a large error in the result. The necessary degree of precision is seldom attained in routine work.

In the method here described, prolonged centrifuging is unnecessary and the difficulties involved in the determination of the increase in specific gravity due to protein in the presence of high salt concentration are obviated. After precipitation with ammonium sulfate, the globulin fraction is treated with trichloroacetic acid in order to dilute the salt. It is then redissolved in dilute sodium hydroxide. The difference between the specific gravity of the resulting solution and that of a blank, prepared from an equal volume of the trichloroacetic acid supernatant similarly treated with sodium hydroxide, is a function of the protein content of the solution. Globulin and albumin are calculated from this difference and the specific gravity of the original serum. This procedure permits the specific gravity measurements to be made on dilute solutions and within the range used on serum itself. Only ordinary care is required in order to obtain reliable and reproducible results.

METHOD

Reagents.—Ammonium sulfate solution prepared by adding 346 Gm. of the anhydrous salt to 1000 Gm. of water.

Trichloroacetic acid solution, 5 per cent.

Sodium hydroxide solution, 2.25 per cent.

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Procedure.—One cubic centimeter of serum is pipetted into a 15 c.c. conical, graduated centrifuge tube, and 10 c.c. of the ammonium sulfate solution is gradually added while shaking. It is then corked and centrifuged at 2500 to 3000 revolutions per minute for ten minutes. The supernatant liquid is drawn off to the 1.0 c.c. mark and discarded. Ten cubic centimeters of 5 per cent trichloroacetic acid are added and thoroughly mixed with the precipitated protein, using a fine glass rod to break up any coagulum which may be packed in the bottom of the tube. The mixture is now centrifuged a second time at the same speed for five minutes. One-half cubic centimeter of the supernatant liquid is transferred to a test tube for use as a blank; the remainder is drawn off to 0.5 c.c. One and one-half cubic centimeters of 2.25 per cent sodium hydroxide are added to both tubes and the resulting solutions are thoroughly mixed. The precipitated protein is best stirred up to form a paste before the addition of sodium hydroxide and then stirred again immediately afterwards until the protein is completely dissolved.

The specific gravities of the two solutions are measured by any suitable method, such as the well-known falling drop.[2, 3]

In making a series of determinations, is it unnecessary to prepare a blank for each analysis. In fact, it has been found possible to use the same blank reading over a period of several weeks, provided the reagents are kept in well-stoppered bottles.

Calculations.—The globulin, G, in Gm. per 100 c.c., is calculated from the formula

$$G = 695\ D - \frac{T}{10} \qquad (1)$$

where D represents the difference in specific gravity between the blank and the protein solution, and T represents the total protein content of the serum in Gm. per 100 c.c.

If measurements are made with the specific gravity pipette, provided with a scale reading directly in percentage of protein as previously described,[4] formula (1) becomes

$$G = 2\ D - \frac{T}{10} \qquad (2)$$

in which D is simply the difference between the readings of the blank and the protein solutions.

Albumin is calculated by subtracting the value of the globulin fraction from the total protein.

Example.—A serum specimen was found by the specific gravity method to have a total protein content of 6.8 Gm. per 100 c.c. When analyzed by this procedure, using the falling drop technique of Barbour and Hamilton,[2] the blank and the protein solutions gave apparent density differences, as read from the alignment chart, of 0.0050 and 0.0091, respectively. Substituting the values in formula (1) gives globulin.

$$G = 695\ (0.0091 - 0.0050) - \frac{6.8}{10} = 2.2 \text{ Gm. per 100 c.c.}$$

With the specific gravity pipette[4] calibrated to read directly in total serum protein percentage, the readings of the blank and the protein solutions were

5.80 and 7.25 when reduced to the same temperature. Using formula (2) the globulin content is

$$G = 2\ (7.25 - 5.80) - \frac{6.8}{10} = 2.2 \text{ Gm. per } 100 \text{ c.c.}$$

Remarks.—In carrying out the foregoing procedure, a centrifuge tube should be used which is known to be accurate at 0.5 and 1.0 c.c.

It is important to add the first 2 c.c. of ammonium sulfate solution to the serum a drop or two at a time with shaking; otherwise, the precipitate may be too voluminous. But with this precaution it will seldom occupy a volume greater than 0.6 c.c. In rare cases it may be necessary to centrifuge for a longer time than ten minutes in order to get a clear supernatant liquid. Turbid solutions may result from failure to tightly cork the centrifuge tube.

For drawing off the supernatant solution after centrifugations, it is convenient to use a tapered glass tube connected to a water-aspirator pump. With suction applied to this tube, the solution may be drawn off rapidly and precisely to the required volume without disturbing the precipitate.

The second centrifugation should not be unnecessarily prolonged, as excessive packing of the protein precipitate retards its solution in sodium hydroxide. At times small gel-like particles which dissolve slowly form upon the addition of alkali. Little or no difficulty from this source is experienced, however, when the mixture is stirred immediately.

Small amounts of the ammonia formed in the reaction of sodium hydroxide with the ammonium salt are unavoidably lost; however, the solution may be allowed to stand for as long as an hour after the addition of alkali without introducing significant error, provided the tube is kept well-corked.

With a little experience a complete serum protein analysis can be made in one-half hour, and four or six determinations can be carried out simultaneously in less than one hour. The method is especially suited to the occasional estimation of serum protein fractions, since the reagents are readily prepared and do not require accurate standardization. With the falling drop method no standard other than that commonly used in measuring the specific gravity of serum is required, while the use of the specific gravity pipette dispenses with the need of a standard solution of any kind.

RELATIONSHIP BETWEEN SPECIFIC GRAVITY AND PROTEIN CONTENT

The specific gravity method for the determination of the total protein content of serum is based upon the investigations of Moore and Van Slyke,[1] Weech, Reeves, and Goettsch,[6] Kagan,[3] and Nugent and Towle,[7] who found that a linear relation exists between specific gravity and amount of protein, having the form

$$\text{Total protein} = K\ (\text{specific gravity} - b)$$

where K is a conversion constant and b represents the specific gravity of serum with protein eliminated. Thus, the specific gravity increment of protein, i.e., the contribution made to the specific gravity by the protein present, is directly proportional to the protein content.

Assuming a similar relationship to exist for protein after having been precipitated and redissolved in sodium hydroxide, it follows that the percentage of

globulin present in the serum in the foregoing procedure may be expressed by the general formula

$$G = \frac{k\ a\ V_f\ D}{(a-b)\ V_s} - \frac{b\ T}{a-b} \qquad (3)$$

in which V_s is the volume of serum used, D is the increase in specific gravity due to the presence of protein, k is a proportionality constant, a is the total volume in cubic centimeters of one-half saturated ammonium sulfate, b is the volume remaining after drawing off the first supernatant solution, V_f is the final volume of the solution after the addition of alkali, and T is the total protein in the original serum in Gm. per 100 c.c.

This equation was tested in an extended series of experiments, using different amounts of pooled human serum analyzed for protein by the Howe[8] technique in conjunction with the macro-Kjeldahl nitrogen method. It was found to hold within rather narrow limits over the range of globulin values likely to be encountered. At the same time, the value of constant, k, was found to be 316.

A comparison of this value for k with the corresponding constant in the formula of Weech and co-workers[6] for total protein in serum, where K is taken as 348, indicates that the apparent specific gravity increment of protein under the conditions in the present procedure is about 10 per cent greater than in the case of pure serum. This difference is probably due to a combination of factors, the most important of which is the retention of a certain amount of trichloroacetic acid by the precipitate. That such a retention occurs was demonstrated by Dulière and Minne.[9] The amount retained was found to be proportional to the weight of protein, however; hence, this factor should not affect the validity of formulas (1-3).

Formula (1) may be obtained by substituting into equation (3) the numerical values designated in the procedure, thus

$$G = \frac{316 \times 11 \times 2\ D}{10} - \frac{T}{10} = 695\ D - \frac{T}{10}$$

Formula (1) reduces to formula (2) when specific gravities are replaced by their percentage protein equivalents as given with the direct-reading pipette.

Small departures from the quantities specified in the procedure are possible if the relationships expressed in equation (3) are taken into account. For example, the final volume of solution may be made 1.5 c.c. instead of 2.0 c.c. by the addition of only 1.0 c.c. of sodium hydroxide solution, provided 1.5 is used as V_f in place of 2.0 in calculating the result. There are practical limits to such variations, however. The addition of too small an amount of alkali, for example, results in failure to completely dissolve the protein precipitate, while an increased amount magnifies the effect of errors in the measurement of specific gravity.

In establishing suitable concentrations and volumes for the solutions involved in this method an experimental study was made of the variations in specific gravity with the composition of different mixtures, both with and without protein present. When 2.25 per cent sodium hydroxide was mixed in different proportions with a solution consisting of 10 volumes of 5 per cent trichloroacetic acid and one volume of one-half saturated ammonium sulfate,

the specific gravity of the resulting solutions varied according to curve ACB shown in Fig. 1. With 1.5 per cent protein present the curve EF was obtained. An amount of protein equivalent to this, for example, appears in the final alkaline solution when a determination is carried out on a one cubic centimeter specimen of serum containing 2.3 per cent globulin and 7.0 per cent total protein. The elevation of any point in EF above the corresponding point in CB represents the specific gravity increment, D, in equations (1-3). This quantity is interpreted as due to and proportional to the protein content of the solution. The line EF also represents the approximate maximum range through which the proportions of the two solutions may be varied in carrying out a determination.

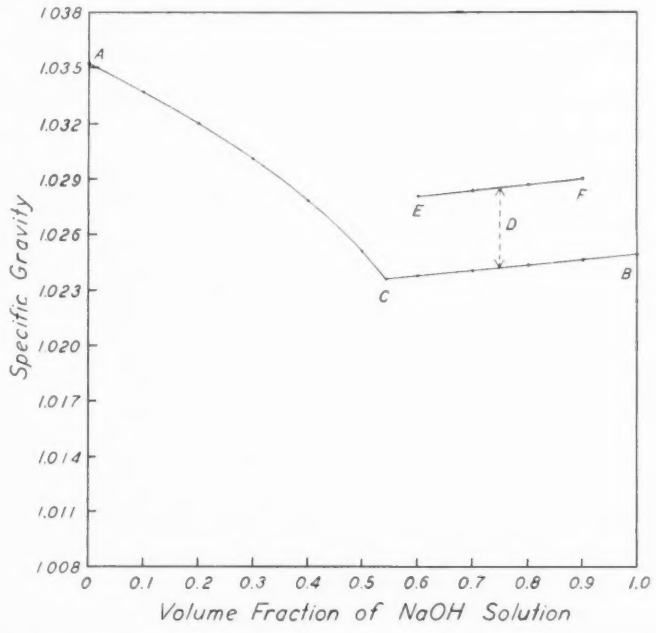

Fig. 1.—Curves representing specific gravity of mixtures of 2.25 per cent NaOH and a solution prepared by adding 10 volumes of 5 per cent trichloroacetic acid to one volume of half saturated ammonium sulfate. Curve ACB, solution containing no protein; curve EF, containing protein. Broken line D represents the increase in specific gravity caused by the presence of protein. Abscissas represent the volume of NaOH solution divided by the sum of the volumes of the two solutions.

RESULTS

Twenty-four determinations by the specific gravity and the Howe-Kjeldahl methods, covering a comparatively wide range of globulin values, are given in Table I. As the method here described is essentially one for the estimation of globulin, values for that fraction only are reported. It will be noted that the correlation between the two methods is close, in fact closer than that usually obtained in a series of total protein determinations. This may be explained in part by the fact that variations in serum constituents, such as salt and glucose, which are soluble in the reagents employed, have no appreciable effect on the result. The standard deviation between the two methods is ±0.14 Gm. per 100 c.c.

SUMMARY

A method for the determination of serum globulin and albumin by specific gravity measurements is described.

TABLE I

COMPARISON OF HOWE-KJELDAHL AND SPECIFIC GRAVITY METHODS FOR SERUM GLOBULIN
Values in Gm. per 100 c.c.

SPECIMEN	HOWE-KJELDAHL	SPECIFIC GRAVITY	SPECIMEN	HOWE-KJELDAHL	SPECIFIC GRAVITY
1	1.7	1.7	13	2.2	2.2
2	3.2	3.1	14	5.1	5.1
3	3.8	3.7	15	1.7	2.0
4	2.1	2.2	16	1.8	1.6
5	4.3	4.0	17	3.0	3.1
6	2.4	2.5	18	1.9	2.0
7	4.3	4.3	19	4.1	4.1
8	3.6	3.6	20	4.9	4.8
9	3.7	4.0	21	5.6	5.6
10	3.7	3.8	22	1.5	1.5
11	2.3	2.3	23	2.7	2.5
12	1.5	1.3	24	2.5	2.5

The globulin is determined by precipitation with one-half saturation of ammonium sulfate, the precipitate being subsequently treated with 5 per cent trichloroacetic acid and redissolved in 2.25 per cent sodium hydroxide. The difference between the specific gravity of the resulting solution and that of a blank prepared from the trichloroacetic acid supernatant liquid represents the amount of protein in the solution, and when used in conjunction with the total protein value gives a basis for calculating both the albumin and globulin fractions.

Any specific gravity method suitable for estimating total serum protein may be employed, and no additional standard is required.

A comparison of the results with those of the Howe-Kjeldahl method is given.

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Erratum

In the article by Drs. Bernardo Sepulveda and Arnold E. Osterberg, entitled "Serum Bilirubin: A Procedure for the Determination of Indirect and Direct Values," in the August, 1943, issue, page 1359, the third line in the third paragraph should read: "bilirubin is removed from the serum by successive extractions with chloroform."

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